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Spectrophotometric Measurements on Ascorbic Acid and Their Use for the Estimation of Ascorbic Acid and Dehydroascorbic Acid in Plant Tissues

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The ultraviolet-light-absorption of ascorbic acid in neutral solutions was described by Herbert, Hirst, Percival, Reynolds & Smith (1933), Baird *et al.* (1934) and Morton (1942). Racker (1952) suggested application of spectrophotometry to enzymic microestimation of ascorbic acid or assay of ascorbic acid oxidases but did not investigate the conditions for such methods or the effects of pH on the absorption spectrum. Effects of pH were studied in detail by Daglish (1951), who described a spectrophotometric assay based on effects of pH on extinction. We have compared enzymic and chemical methods for estimating ascorbic acid and dehydroascorbic acid by spectrophotometric and visual-titration methods. Molar extinction coefficients of L-ascorbic acid found in this work were higher than those recorded by all previous workers except Lawendel (1956, 1957).

The spectrophotometric method we now describe was designed to estimate ascorbic acid in as little as 10 mg. of plant tissue. Between 1 and 75μg. of ascorbic acid or dehydroascorbic acid can be determined in 3 ml. of extract, 2μg. within about 5% and 10–20μg. within about 2% of the amount present. Non-enzymic methods of comparable sensitivity, with 2:6-dichlorophenolindophenol (Bessey, 1938; Glick, Alpert & Stecklein, 1953), are liable to interference, especially by reducing agents other than ascorbic acid. Chromatographic separation from reductones and thiols has been used by Chen, Isherwood & Mapson (1953) and by Strohecker, Heimann & Matt (1955). These methods also separate L-ascorbic acid from D-arabo- and D-gluco-ascorbic acid, but are lengthy and result in substantial losses with some plant tissues.

The estimation of dehydroascorbic acid with an indophenol dye by the difference in ascorbic acid found before and after reduction with hydrogen sulphide (Bessey, 1938) or with homocysteine (Hughes, 1954, 1956) is dependent on no other reducing compounds being produced by these treatments. We have compared the effects of reduction by hydrogen sulphide or homocysteine on the determination of dehydroascorbic acid by independent enzymic or chemical means.

EXPERIMENTAL

Materials

L-Ascorbic acid (British Drug Houses Ltd.) was found to be 99·4% pure by titration with KIO_3 in 4N-HCl in the presence of $CHCl_3$ (1 drop) to detect free I_2 at the end point

(Andrews, 1903). The melting point was 188° (uncorr.) with decomposition.

Solutions of dehydroascorbic acid were prepared as required by oxidation of ascorbic acid with bromine water in 2% (w/v) HPO_3 as described by the Association of Vitamin Chemists (1947). The solution was aerated to remove excess of Br_2. Measurements of light-absorption at 265 mμ before and after oxidation, and on subsequent reduction by H_2S at pH 3·0–3·5 (Levenson, Rosen & Hitchings, 1951) or by homocysteine at pH 6 (Hughes, 1956) were compared with titration by 2:6-dichlorophenol-indophenol reagent; they showed that both oxidation and the subsequent recovery of ascorbic acid by reduction were complete. A sample of solid dehydroascorbic acid (Sigma Chemical Co.) was found to be 80% pure by these tests.

Dihydroxyfumaric acid (Hartree, 1953), called dihydroxymaleic acid in earlier literature, was prepared by peroxidation of tartaric acid in the presence of ferrous sulphate (Powers, Tabakoglu & Sable, 1955). The product was recrystallized from aqueous methanol and dried *in vacuo*, m.p. 155° (uncorr.) in agreement with Fenton (1894); light-absorption maximum was 290 mμ (Hartree, 1953; Baraud, 1954). The molar extinction coefficient (ε) at 290 mμ immediately after dissolution in water was $7·8 \times 10^3$, which is about 15% lower than the value (9×10^3) given by Hartree (1953).

Metaphosphoric acid solutions were freshly prepared by dilution of a 20% (w/v) solution. Copper was removed by extraction with 0·05% (w/v) diphenylthiocarbazone (dithizone) in CCl_4 (Parks, Hood, Hurwitz & Ellis, 1943). The effectiveness of this method was tested by adding known amounts of Cu^{2+} ions to a copper-free sample of HPO_3. Trisodium phosphate was free from copper or other metals that react with dithizone. Solutions of 2:6-dichlorophenolindophenol were standardized by rapid titration against pure ascorbic acid in 2% (w/v) HPO_3 (Harris & Olliver, 1942). Water was obtained by treatment of rain water through several ion-exchange resins including Bio-Deminrolit (Hewitt, 1952). It contained less than 0·0005 p.p.m. of Cu^{2+} ions. Hydrochloric acid (A.R. grade) was redistilled as a 6N-solution from Pyrex glass before use.

Cucumber ascorbic acid oxidase was obtained from the cell sap of cucumber fruits thawed after storing indefinitely in the frozen state at −18°. The fraction precipitated with ammonium sulphate between 65 and 75% saturation was used. This was stable for several days in solution at 0°. Addition of the enzyme to solutions of dihydroxyfumaric acid had no effect on the slow decrease in extinction of the latter at 290 mμ (about 4%/hr. for an aqueous 20 μM-solution). Dihydroxyfumaric acid-oxidase activity was therefore apparently absent. No dehydroascorbic acid-reductase activity was detected. The enzyme solutions usually contained 2 mg. of protein/ml. and the addition of 0·05 ml. of this solution to 3 ml. achieved the oxidation of 90 μg. of ascorbic acid present at a concentration of 30 μg./ml. Preparations should have an activity of at least 4 Snow & Zilva units (Snow & Zilva, 1938), corresponding to an initial change in extinction at 265 mμ of 0·2/min. in 3 ml. of solution containing 15–60 μg. of ascorbic acid.

Estimations of pH were made with a glass electrode and direct-reading pH meter (Electronic Instruments Ltd.). Ultraviolet-absorption measurements were made with a Hilger Uvispek spectrophotometer in 1 cm. silica cells and all readings were corrected for blank absorption.

Spectrophotometric determination of ascorbic acid and dehydroascorbic acid

Standard solutions were prepared by dissolving 40–100 mg. of ascorbic acid in 100 ml. of 2% (w/v) dithizone-extracted metaphosphoric acid. Suitable portions were taken as soon as possible, the pH was adjusted to 6·8 with a predetermined amount of trisodium phosphate and the volume made up to a convenient amount before measurement of absorption at 265 mμ. Fig. 1 shows the agreement between extinction values for ascorbic acid and for corresponding amounts of dehydroascorbic acid (prepared as described above) after subsequent reduction with homocysteine (as described below). The recovery after oxidation and subsequent reduction is not affected by using unpurified metaphosphoric acid.

Plant samples (0·05–1 g.) were ground immediately in 10–30 times their weight of 2% (w/v) fresh copper-free metaphosphoric acid and a small amount of Pyrex-glass powder in a glass homogenizer (Townson and Mercer Ltd.) attached to a stirrer motor controlled with a rheostat. The extract and washings were transferred to 5, 10 or 25 ml. flasks and 0·21 M-trisodium phosphate solution was added (0·66 vol. relative to the volume of HPO_3 used). The volume was made up with 2% (w/v) metaphosphoric acid–0·21 M-trisodium phosphate (3:2, v/v). The pH should be 7·3–7·4.

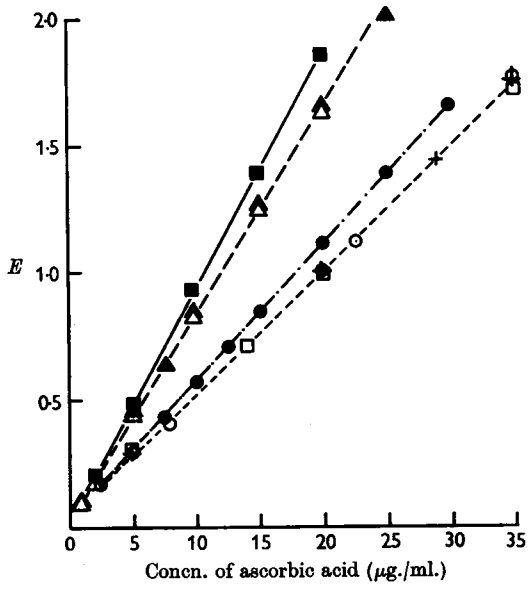

Fig. 1. Relationships between light-extinction (1 cm. depth) and concentrations of ascorbic acid (μg./ml.) in solutions of different composition: ■, at 265 mμ in purified 2% (w/v) metaphosphoric acid, neutralized to pH 6·8; ▲, at 265 mμ in unpurified metaphosphoric acid, neutralized to pH 6·8; △, as ▲ after oxidation with bromine water and subsequent reduction with homocysteine; ●, at 245 mμ in 2% (w/v) unpurified metaphosphoric acid, pH < 2; □, at 250 mμ in 2% (w/v) purified metaphosphoric acid, neutralized to pH 6·8; ○, at 250 mμ in 2% (w/v) metaphosphoric acid, pH < 2; +, at 250 mμ in water.

The extract was centrifuged for 3 min. at 3000*g* and the extinction (*A*) was measured at 265 mμ against a blank containing the metaphosphoric acid–trisodium phosphate mixture. Ascorbic acid-oxidase solution (0·05 ml.) was added to each cell and the extinction (*B*) was read after 15 min. To a similar portion was added 0·05 ml. of a solution containing 30 mg. of homocysteine/ml., which provides a molecular ratio of homocysteine to dehydroascorbic acid of between 50:1 and 250:1. The extinction (*C*) was read after 15 min. against a blank containing homocysteine and the metaphosphoric acid–trisodium phosphate mixture. The concentration of ascorbic acid (μg./ml.) was calculated from 10·80 ($A - 1{\cdot}02B$), and that of dehydroascorbic acid from 10·65 ($1{\cdot}02C - A$). The factors are based on $\epsilon\ 1{\cdot}65 \times 10^4$ for ascorbic acid and $\epsilon\ 2{\cdot}3 \times 10^2$ for dehydroascorbic acid at 265 mμ, their molecular weights and the dilutions in the cells. The initial extinction (*A*) varies greatly with plant species.

For 10–50 mg. samples, hydrogen sulphide was used in place of homocysteine. The sample was ground in 1·8 ml. of 2% (w/v) metaphosphoric acid and the extract transferred to a centrifuge tube. The homogenizer was washed out with 1·2 ml. of 0·21 M-trisodium phosphate solution and the extract and washings were centrifuged. The supernatant fluid was transferred as completely as possible to a 1 cm. absorption cell and reading *A* was made. Purified metaphosphoric acid (0·2 ml. of 40%, w/v) was added to lower the pH to 3·0–3·5 and hydrogen sulphide passed for 15 min., followed by N_2 for 45 min. Solid trisodium phosphate ($Na_3PO_4,12H_2O$) (copper-free) was added and dissolved with the aid of a fine glass rod to raise the pH to 7·0–7·4, and reading *C* was made. Enzyme solution (0·05 ml.) was stirred in and reading *B* was made after 15 min. Calculations are similar to those for the larger samples, but volume changes involved during transfer of the extract and addition of metaphosphoric acid, sodium phosphate and enzyme require the use of the factor 1·10 in place of 1·02.

RESULTS

Ultraviolet-light absorption of ascorbic acid solutions

Effects of pH. The absorption maximum of ascorbic acid was found at 265 mμ at pH > 6·8 and at 244 mμ at pH < 1·5, in agreement with Herbert *et al.* (1933), Baird *et al.* (1934), Morton (1942) and Daglish (1951). In unbuffered solutions the wavelength of the absorption maximum depends on concentration; it occurs at 264 mμ with 5μg./ml. (pH 4·70), at 256 mμ with 15μg./ml. (pH 4·35) and at 249 mμ with 35μg./ml. (pH 4·10), and the departure from Beer's law is considerable. In buffered solutions the wavelengths of the maxima at the pH values > 6·8 and < 1·5 are independent of the concentration of ascorbic acid. The light-extinction of ascorbic acid solutions in 2% (w/v) HPO_3, or in purified metaphosphate neutralized to pH 6·8, obeys Beer's law over the range 0–20μg./ml. (or over) as shown in Fig. 1. In unpurified, neutralized metaphosphoric acid, the deviation from Beer's law is negligible but the extinction may be low. The relationship between the values for $\lambda_{max.}$ and pH suggest a p*K* for ascorbic acid of about 4·25. This value is only slightly higher than that (4·15) found by Daglish (1951).

Isosbestic point. Three sets of absorption curves were obtained for different concentrations of ascorbic acid in 2% (w/v) HPO_3, in water and in metaphosphoric acid neutralized to pH 6·8. Their intersection at about 250 mμ confirmed the findings of Daglish (1951). An alternative and sensitive method for determination of the isosbestic point was provided by observation of the wavelength at which changes in pH had no effect on light-absorption values, since extinction at the isosbestic point is independent of the proportions of ionized and non-ionized forms determined by pH. The wavelength determined by this method was also 250 mμ, as shown in Fig. 2. Extinction at the isosbestic point obeyed Beer's law up to 35μg./ml. (or over) (Fig. 1), regardless of the wavelengths at which the absorption maxima occurred.

Molar extinction coefficients. Widely different values have been reported for the extinction of solutions of ascorbic acid (Lawendel, 1957). The values for ε found by us at first, from several determinations on different occasions, ranged between $1{\cdot}33 \times 10^4$ and $1{\cdot}55 \times 10^4$ with a mean of $1{\cdot}45 \times 10^4$ at 265 mμ and pH 6·8, and between $9{\cdot}95 \times 10^3$ and $1{\cdot}08 \times 10^4$ with a mean of $1{\cdot}03 \times 10^4$ at 245 mμ and pH 1–2. These values were greater than those commonly accepted (Baird *et al.* 1934; Morton, 1942; Brode, 1946) and similar to those ($1{\cdot}5 \times 10^4$, 265 mμ and $9{\cdot}6 \times 10^3$, 245 mμ) given by Daglish (1951).

While this work was in progress, Lawendel (1956, 1957) reported that when ascorbic acid is dissolved in media containing sorbitol at neutral

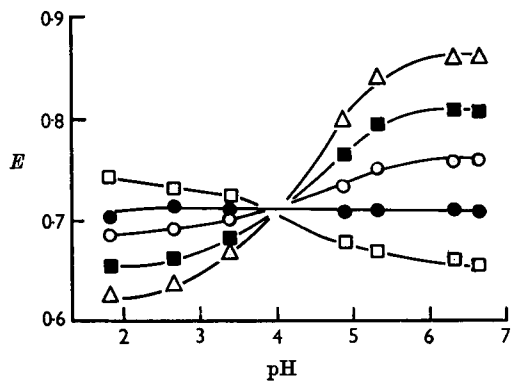

Fig. 2. Relationships between pH and light-extinction (1 cm. depth) of solutions of ascorbic acid (14μg./ml.) at wavelengths near the isosbestic point: △, at 253 mμ; ■, at 252 mμ; ○, at 251 mμ; ●, at 250 mμ; □, at 249 mμ.

Table 1. *Extinction coefficients of ascorbic acid in aqueous solutions of different* pH *and composition*

pH	Medium	Wavelength (mμ)	$10^{-4}\,\epsilon$	$E^{1\%}_{1\,cm.}$
6·8 or above	Neutralized Purified HPO_3	265 (max.)	1·65	936
6·8	Neutralized Unpurified HPO_3	265 (max.)	1·45 Range: 1·35–1·55	823 766–880
1·5 or below	Purified HPO_3 or HCl	244 (max.)	1·05	595
< 2·0	Unpurified HPO_3	245	1·03 Range: 0·99–1·08	585 562–613
pH-independent	HPO_3, water only or neutralized HPO_3	250 (isosbestic point)	0·885	503

pH, higher extinction values than those previously recorded are obtained and the extinction in 'copper-free' media from which oxygen is excluded is similar to that observed in the presence of sorbitol. The values reported by Lawendel were $E^{1\%}_{1\,cm.}$ 916–940 at 265 mμ and pH 6·5, and 676–695 at 245 mμ in 0·1N-HCl. These correspond to ϵ $1{\cdot}612$–$1{\cdot}653 \times 10^4$ and $1{\cdot}190$–$1{\cdot}222 \times 10^4$ respectively.

As our results appeared low we sought to confirm Lawendel's observations with sorbitol, but without success. Tests with dithizone showed that variable trace amounts of copper were present in the metaphosphoric acid solutions and that the latter could be purified by dithizone extraction before adjustment of the pH to 6·8. By using dithizone-purified metaphosphoric acid values between $1{\cdot}63 \times 10^4$ and $1{\cdot}66 \times 10^4$ at 265 mμ were obtained under aerobic conditions at pH 6·8 without sorbitol. The calibration curve in Fig. 1 prepared by dissolving ascorbic acid in purified metaphosphoric acid before neutralization and dilution gave a value ϵ $1{\cdot}655 \times 10^4$ at 265 mμ and pH 6·8.

Repeated measurements between 243 and 245 mμ of light-absorption by solutions containing 5–35 μg. of ascorbic acid/ml. in purified un-neutralized metaphosphoric acid or in either 0·1N- or N-HCl (copper-free) produced values for ϵ between $1{\cdot}01 \times 10^4$ and $1{\cdot}06 \times 10^4$, i.e. $E^{1\%}_{1\,cm.}$ was 573–602 and never approached values of 676–695 (Lawendel, 1957). Measurements with two spectrophotometers [Hilger Uvispek and Optika (UK)] indicated that the absorption maximum is close to 244 mμ, where the extinction is about 1·5% higher than at 245 mμ. Our measurements are recorded in Table 1. The extinction at 250 mμ is only about 4% greater than the value observed by Daglish (1951).

Estimation of ascorbic acid and dehydroascorbic acid

Comparisons between alternative procedures. Examples of results for small samples or quantities, for which H_2S was used, are given in Table 2. Results given in Table 3 show no consistent differences between homocysteine and H_2S as reducing agents or between the indophenol titration and the enzymic spectrophotometric methods. Use of bromine water to oxidize ascorbic acid in extracts caused errors due to changes in other substances absorbing light at 265 mμ. The treatment with H_2S sometimes resulted in turbidity, which caused errors unless the solution was filtered.

The control of pH is important in both homocysteine- and H_2S-reduction procedures. Levenson, Rosen & Hitchings (1951) found that reduction by H_2S is reversible, rapid and complete at pH 3·5, but that other irreversible reactions become significant below pH 3·0. The reduction with homocysteine is relatively slow at pH 6·5. We found that at pH 6·5 the reaction between homocysteine and dehydroascorbic acid in molar ratio 40:1 was only 90% complete in 30 min. at room temperature, whereas at pH 7·3 and above, with a molar ratio of only 10:1, the reaction was complete in less than 15 min. This is in agreement with observations at pH 7·2 by Hughes (1956). The pH values should not, however, exceed 7·5, as ascorbic acid is unstable when the pH is near 8.

Effect of dihydroxyfumaric acid. Dihydroxyfumaric acid interferes with the estimation of ascorbic acid by the indophenol method and, according to Snow & Zilva (1938), dihydroxyfumaric acid is oxidized by crude cucumber-oxidase extracts unless these are dialysed. As dihydroxyfumaric acid absorbs light at 265 mμ (Hartree, 1953; Baraud, 1954) it was necessary to test whether interference occurred. Cucumber oxidase fractionated with ammonium sulphate as described above produced no change in the slow rate of decrease in extinction at 265 or 290 mμ of solutions of dihydroxyfumaric acid prepared by us, and it is concluded from this that this compound does not interfere in the spectrophotometric estimation of ascorbic acid when the partially purified cucumber oxidase is used.

Recovery of ascorbic acid and dehydroascorbic acid. Addition of ascorbic acid or dehydroascorbic acid to metaphosphoric acid extracts show that satis-

factory recoveries were obtained (Table 4). When varying amounts of ascorbic acid were added to samples of fresh tissue immediately before grinding in metaphosphoric acid or when tissues were ground in metaphosphoric acid containing known concentrations of ascorbic acid there were losses of 20–35% of the amounts added. These losses were fully recovered as dehydroascorbic acid (Table 5). The ratio of ascorbic acid to dehydroascorbic acid in extracts of tissues to which no ascorbic acid had been added was higher than when ascorbic acid was added to tissues before grinding. Addition of ascorbic acid after grinding in metaphosphoric acid, or after centrifuging, resulted in recoveries of 94 and 96% respectively. Several alternative extraction methods were tried. These included the use of 10% (w/v) purified metaphosphoric acid, or of 2% or 10% (w/v) trichloroacetic acid saturated with ether, or plunging samples into boiling ethanol. None of these materially altered the proportions or amounts of ascorbic acid and dehydroascorbic acid subsequently found. The use of metaphosphoric acid solutions saturated with ethylenediaminetetra-acetic acid had no effect on the recovery of ascorbic acid added before grinding.

The method of estimation recently described by Barker & Mapson (1959), in which extraction is carried out under a stream of N_2 at $-3°$ in 4% (w/v) metaphosphoric acid, was compared with the one used here (Table 6). The differences in the ascorbic acid and dehydroascorbic acid contents found by the two methods were small.

DISCUSSION

The results obtained by us and by Lawendel (1956, 1957) show that the molar extinction coefficient of ascorbic acid at 265 mμ is much

Table 2. *Estimation of ascorbic acid and dehydroascorbic acid in separate samples of cauliflower leaf*

All extinction measurements were made on the same portion of solution for each sample and calculated from the formulae 10·80 ($A - 1·1B$) for ascorbic acid and 10·65 ($1·1C - A$) for dehydroascorbic acid.

		Ascorbic acid		Dehydroascorbic acid	
Wt. of tissue (mg.)	$E_{265\ m\mu}$	μg./ml. of extract	μg./g. of leaf	μg./ml. of extract	μg./g. of leaf
13·4 (in 3·0 ml.)	*A* 0·319	—	—	—	—
	B 0·098	2·29	513	—	—
	C 0·315	—	—	0·29	65
11·7 (in 3·0 ml.)	*A* 0·236	—	—	—	—
	B 0·074	1·68	431	—	—
	C 0·235	—	—	0·21	54
360 (in 100 ml. (i)*	*A* 0·418	—	—	—	—
	B 0·220	2·14	595	—	—
	C 0·425	—	—	0·53	147
(ii)	*A* 0·418	—	—	—	—
	B 0·224	2·12	589	—	—
	C 0·423	—	—	0·51	141

* (i) and (ii) are determinations on duplicate 3 ml. samples drawn from 100 ml. of extract of 360 mg. of the tissue corresponding to 10·8 mg. in 3·0 ml.

Table 3. *Estimation of ascorbic acid and dehydroascorbic acid by different procedures*

Values are given as μg./g. fresh wt. in separate experiments.

Fraction	Procedure	Cauliflower	Tomato
Ascorbic acid	Spectrophotometric	592 658 791 461 584	912
	Indophenol titration	603 648 778 474 598	909
Dehydroascorbic acid	Homocysteine; spectrophotometric	125 133 241 89 155	73
	H_2S; spectrophotometric	125 140 230 91 159	—
	Homocysteine; indophenol titration	125 138 225 95 176	—
	H_2S; indophenol titration	132 143 236 87 165	81
Total ascorbic acid + dehydroascorbic acid by addition*	Spectrophotometric (mean)	717 795 1030 552 741	985
	Indophenol titration (mean)	732 789 1010 565 769	990
Total ascorbic acid + dehydroascorbic acid by separate estimation†	H_2S; spectrophotometric	709 784 994 556 745	—

* The mean values for dehydroascorbic acid estimated by homocysteine or H_2S were used in the additions.

† Dehydroascorbic acid was reduced by H_2S before the total, as ascorbic acid, was estimated as described in the text for samples of 0·05–1 g.

Table 4. *Recovery of ascorbic acid or dehydroascorbic acid added to extracts of tomato- or cauliflower-leaf tissues*

	Added to extract (μg./ml.)	Found in extract (μg./ml.)	Expected (μg./ml.)	Recovery of added acid (%)
	Tomato leaf (40 mg./125 ml.)			
Ascorbic acid	Nil	0·80	—	—
	0·5	1·33	1·30	106
	1·0	1·72	1·80	92
	2·0	2·80	2·80	100
	4·0	4·74	4·80	99
	Tomato leaf (100 mg./125 ml.)			
Ascorbic acid	Nil	2·28	—	—
	6·0	8·10	8·28	97
	9·0	11·4	11·3	101
	Tomato leaf (100 mg./125 ml.)			
Dehydroascorbic acid	Nil	0·15	—	—
	1·0	1·13	1·15	98
	3·0	3·07	3·15	97
	5·0	5·01	5·15	97
	Tomato leaf (100 mg./42 ml.)			
Dehydroascorbic acid	Nil	0·53	—	—
	0·5	1·03	1·03	100
	1·0	1·56	1·53	102
	3·0	3·26	3·53	91
	5·0	5·26	5·53	95
	Cauliflower leaf (70 mg./83 ml.)			
Ascorbic acid	—	1·33	—	—
	2·48	3·85	3·81	102
	4·96	6·43	6·29	103
	9·92	11·1	11·3	99

Table 5. *Recovery of added ascorbic acid as ascorbic acid and dehydroascorbic acid*

Ascorbic acid was added to 250 mg. of cauliflower leaf before it was ground in 25 ml. of copper-free metaphosphoric acid.

Concn. in leaf extracts without added ascorbic acid (μg./ml.)			Found in leaf extracts with added ascorbic acid (μg./ml.)			Recovery of added ascorbic acid (%)	Ascorbic acid/dehydroascorbic acid in extracts	
Ascorbic acid	Dehydroascorbic acid	Ascorbic acid added (μg./ml.)	Ascorbic acid	Dehydroascorbic acid	Total		Without added ascorbic acid	With added ascorbic acid
7·15	1·54	5	10·4	3·20	13·6	65	4·64	3·26
6·70	1·12	10	14·5	3·10	17·6	78	6·01	4·70

Table 6. *Comparison of results of extraction by the method of Barker & Mapson* (1959) (1) *and the method described in the text* (2)

Ascorbic acid and dehydroascorbic acid were determined spectrophotometrically in different samples of cauliflower leaf weighing 250 mg.

Sample	I		II		III	
Method	1	2	1	2	1	2
Ascorbic acid (μg./g.)	775	763	663	640	643	631
Dehydroascorbic acid (μg./g.)	80	74	58	61	100	102
Ascorbic acid/dehydroascorbic acid	9·7	10·3	11·4	10·5	6·4	6·2

higher than that previously recorded. The maximum value recorded here is $1{\cdot}66 \times 10^4$. Since values tend to be low, due to oxidation, the value ϵ $1{\cdot}65 \times 10^4$ at 265 mμ and pH 6·8 or above, which agrees with that reported by Lawendel, is accordingly proposed. We are unable, however, to confirm Lawendel's figure for ϵ at 245 mμ in 0·1N-HCl or in metaphosphoric acid or under nitrogen in acid, and we propose the value ϵ $1{\cdot}05 \times 10^4$ at 244 mμ and pH 1·5 or below. This is close to values reported by several workers, who nevertheless recorded relatively low values for corresponding measurements at 265 mμ and pH > 6·5. In the work described here care was taken to exclude as far as possible traces of copper and other heavy metals from water and reagents. Glass-distilled water of probably low copper content was used by many who recorded low values for ϵ at 265 mμ in earlier work. However, the buffer reagents used to produce the pH values above 6·5 were often not purified, and heavy-metal-catalysed oxidation of ascorbic acid probably occurred. By using metaphosphoric acid and trisodium phosphate which were free of copper, catalysis of oxidation by Cu^{2+} ions was avoided. No changes in extinction were observed over several hours for plant extracts in 2% (w/v) purified metaphosphoric acid (pH 1·5). At pH 7 in neutralized purified metaphosphoric acid a slow decrease in extinction at 265 mμ was observed, amounting only to 1% in the first 15 min. and thereafter to about 0·5%/hr. over 24 hr. The error to be expected from these changes during the period of the determination is therefore negligible. It might be concluded that as copper and oxygen have greater effects on loss of ascorbic acid at pH values of about 6·5–7·0 than in acid solutions, light-extinction values would be less inaccurate at 245 than at 265 mμ at the appropriate pH values. We are also unable to confirm Lawendel's observations on the effect of sorbitol. It is possible that his sample contained a copper-chelating impurity which served to protect ascorbic acid from catalytic oxidation.

The analytical method described above has the advantage of greater specificity than methods based on indophenol and is at least as sensitive and as easy to apply. The change in extinction at 265 mμ (pH 7·3) due to oxidation with cucumber oxidase is about double that produced at 265 mμ by changing the pH from 3·0 to 4·6; the present method is thus proportionately more sensitive than that proposed by Daglish (1951), which was based on this effect of pH.

The extent of interference by reductones and reductic acid has not been established. The absorption maximum of reductone is at 285 mμ at pH 5·7, according to Euler, Hasselquist & Ceder (1953), who gave no data for extinction in relation to concentration. The results obtained by Snow & Zilva (1938) in a comparative study of the oxidation of ascorbic acid, reductone and reductic acid by ascorbic acid oxidase indicate the extent to which possible interference by reductone may occur and how it may be minimized. They found that the rates of oxidation of reductone and of reductic acid by the oxidase were much less than those of equivalent concentrations of ascorbic acid, and further that the differences in these rates become more marked as the concentrations of the respective substrates are decreased. Their data indicate that at concentrations of ascorbic acid of 10–20μg./ml. the rate for ascorbic acid should be at least 20 times that for equivalent concentrations of reductone or reductic acid. Enzyme concentration also affects rates of oxidation of the substrates in a differential manner: increasing enzyme concentration increases the rate of oxidation of ascorbic acid relative to that of equivalent concentrations of reductone. For this reason enzyme preparations of the activity specified in the Experimental section should be used and the reaction time kept to a minimum (usually 10–15 min.). Under these conditions, where ascorbic acid concentrations of the order of 0·1 mM or less are involved, the error caused by the presence of equivalent concentrations of reductone or reductic acid might be as low as 5%.

Mapson (1958) queried whether any existing method provided a valid estimate of the proportions of ascorbic acid and dehydroascorbic acid in intact cells. It would appear from Tables 4 and 5 that added ascorbic acid is converted into dehydroascorbic acid during extraction, and that this reaction occurs to a relatively greater extent than in unsupplemented control extracts. Whether cellular ascorbic acid is also converted into dehydroascorbic acid during grinding remains unsettled. In attempts to elucidate this point, Barker & Mapson (1959) investigated effects of different extraction procedures on the ratio ascorbic acid/dehydroascorbic acid, especially with reference to the apparent content of dehydroascorbic acid. The method finally evolved by them, to which reference was made at the end of the preceding section, was designed to minimize the production of dehydroascorbic acid as an artifact resulting from enzymic oxidation, by exclusion of air and extraction at a low temperature. The comparisons shown in Table 6, which were made at the suggestion of Dr L. W. Mapson, showed no consistent or notable differences in the results obtained by the two methods. Although we consider that atmospheric oxidation catalysed by ionic copper introduced by the extracting medium can be discounted as the cause of the production of dehydroascorbic acid, it is possible that copper compounds extracted from

the plant tissues may catalyse the oxidation of ascorbic acid, at the time of cell rupture. Attempts to exclude atmospheric oxygen by saturating the extracting medium with nitrogen and flushing with nitrogen during extraction in an open vessel (Barker & Mapson, 1959) may not entirely prevent the access of oxygen. The oxygen consumption involved in the production of the amounts of dehydroascorbic acid recorded in Table 6 would in fact amount to only about 3μl. for 250 mg. of leaf tissue if hydrogen peroxide were produced and only 1·5μl. if peroxide were not formed. The relative stability of ascorbic acid in purified metaphosphoric acid extracts in air and the similarity in the amounts of dehydroascorbic acid obtained by the two methods compared in Table 6 suggest, however, that if dehydroascorbic acid is produced as an artifact in plant extracts, through the activity of ascorbic oxidase or indirectly of phenol oxidase enzymes, the reaction occurs for a short period only, at the time of cell rupture. In practice this reaction will be minimized by avoiding mechanical injury of the tissues as far as possible before extraction. 'Pickling' for a short time before extraction in an acid reagent which chelates copper and also penetrates rapidly might be advantageous, but has not been tried.

SUMMARY

1. L-Ascorbic acid at pH 6·8 or over has $\epsilon_{max.}$ (265 mμ) of 1·65–1·655 × 10^4. This value agrees with that of Lawendel (1956) but an effect of sorbitol reported by him could not be confirmed.

2. At pH between 1·0 and 2·0, in metaphosphoric acid or in hydrochloric acid, $\epsilon_{max.}$ (244 mμ) is 1·05 × 10^4. This value is about 15% lower than that reported by Lawendel.

3. An isosbestic point which is independent of pH occurs at 250 mμ where ϵ is 8·85 × 10^3.

4. Light-extinctions obey Beer's law at 265 and 245 mμ for concentrations of ascorbic acid up to 35μg./ml. in buffered solutions of pH 6·8 or above and pH 1–2 respectively, and at 250 mμ regardless of these pH limits.

5. A spectrophotometric method for the estimation of small amounts of ascorbic acid and dehydroascorbic acid in extracts of leaf tissues is described.

6. Satisfactory agreement was obtained between (*a*) indophenol titrations and (*b*) spectrophotometric changes resulting from oxidation by cucumber oxidase, and subsequent reduction of dehydroascorbic acid by either homocysteine or hydrogen sulphide.

7. Good recoveries of ascorbic acid and dehydroascorbic acid were obtained when small amounts were added to extracts after grinding. When such additions were made before extraction, losses of ascorbic acid up to 35% occurred and these were recovered as dehydroascorbic acid. The conversion of ascorbic acid into dehydroascorbic acid during extraction is greater for added ascorbic acid present in an extracellular location than for that present within the cells.

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