Stability of Ascorbic Acid in Extracts of Iris Leaves

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The highest average value for the ascorbic acid content of freshly harvested vegetables in Olliver's (1954) list is 1.9 mg./g. fresh wt. for parsley (*Carum petroselinum*); in the *Handbook of Biological Data* (1956) the highest value is 1.36 for turnip greens (*Brassica rapa*). Morgan (1942) found 2.8 mg./g. in fresh parsley leaves. We have found the average value for several batches of lucerne (*Medicago sativa*) to be 2.4 mg./g. Other high values in published tables are those of Bicknell & Prescott (1953) for nasturtium (*Tropaeolum majus*) leaves, 2.0–4.65 mg./g., and of Porotnikoff (1954) for kale, 1.84 mg./g. The present paper shows that leaves of the iris have a higher content of ascorbic acid than any of those mentioned above and also that they have an exceptionally low oxidase activity.

METHODS

Determination of ascorbic acid. Samples (about 0.5 g.) of fresh iris leaf were obtained by cutting thin slices across five places along each of several leaves. Each sample was quickly weighed and ground with quartz in beakers (see Booth, 1957) under 3 ml. of freshly prepared 20% (w/v)

metaphosphoric acid. Water (15 ml.) was added and the extract filtered. Duplicate portions of the filtrate were titrated by the method of Bessey (1938) in which excess of 2,6-dichlorophenolindophenol was added and the unreduced dye was measured in a photoelectric absorptiometer.

Two cultivated varieties of *Iris germanica* were used for all the experiments except for five results in Table 1.

Test for oxidase. The ascorbic acid in a sample of leaf was determined as described above, except that 3 ml. of 0.1 M-sodium phosphate buffer, pH 6.5, were added after the metaphosphoric acid. A similar sample was ground to a paste with quartz under 3 ml. of the buffer, and kept, with occasional stirring, at room temperature for a measured time. After the addition of metaphosphoric acid and water the ascorbic acid was determined.

Water content. This was determined by loss of weight after heating at 100° for 4 hr.

RESULTS

Ascorbic acid content. Results for several batches of iris leaf are given in Table 1. Most of the values were near to 4 mg./g. fresh wt. Moisture determinations made on a few samples averaged 87%. Hence the ascorbic acid content was 31 mg./g. based on dry wt.; or 4.6 mg./ml. if expressed as concentration in plant water as suggested by Hagène (1959).

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Table 1. *Ascorbic acid content of leaves of Iridaceae*

Each value is the mean of at least duplicate determinations on a different batch of leaves analysed on a different day.

Type	Colour of flower	Month of collection	Ascorbic acid (mg./g. of fresh leaf)
<i>Germanica</i> cv.	Purple	March	3.89
	Purple	April	4.55
	Purple	May	3.30, 3.85, 4.30, 3.81, 3.74, 4.46
	Purple	June	4.17, 3.62, 4.32, 4.08
	Purple	July	2.73, 3.33
	Purple	August	4.23, 3.59
	Purple	September	5.30, 3.19
	Purple	November	2.45
	Brown	April	3.99
	Brown	June	4.27
	Brown	October	2.42
<i>Chaemeris</i>	Yellow	May	4.96
	Purple	May	5.08
<i>Xyphium</i> cv.	Yellow	May	4.32
<i>Xyphioides</i>	Blue	July	5.26
<i>Gladiolus</i> cv.	Orange	May	5.04
General average			4.01

Concentration gradient. From *I. germanica* plants 15 leaves about 70 cm. long were collected in July, and samples were taken at five equidistant places from near the base to near the apex. Similar samples were taken from another batch of leaves 39 cm. long. The average ascorbic acid concentration was 3.74 mg./g. fresh wt. in the longer leaves, and 2.93 in the shorter. The dry matter averaged 7.9% near the base through 9.8, 12.1, 15.3 to 19.6% near the tip. The average ascorbic acid content of long and short leaves was 38.2 mg./g. dry wt. near the base through 36.5, 29.2, 23.2 to 15.0 near the apex. The ascorbic acid content/g. fresh wt. scarcely changed along the leaf. Hagène (1959), using *I. foetidissima* and *I. pseudacorus*, found an ascorbic acid gradient increasing from base to apex, calculated on fresh weight.

Determination of ascorbic acid by the hydrazine method. Triplicate samples of iris leaf were extracted with 10% acetic acid in 5% metaphosphoric acid. Portions of each extract were used for the determination of total ascorbic acid by the 2,4-dinitrophenylhydrazine method of Roe & Oesterling (1944). The average of the triplicates was 4.43 mg./g. Portions of each extract were also analysed by the indophenol method. The average of the determinations was 3.99 mg./g. The experiment was repeated with another batch of leaves when values of 4.64 and 4.55 respectively were found.

Determination of ascorbic acid by the homocysteine method. Portions of a metaphosphoric acid extract of iris leaf titrated with indophenol dye indicated an ascorbic acid content of 3.49 mg./g. From the same extract 20 ml. was shaken with 0.25 g. of activated charcoal (Norite), to oxidize the ascorbic acid, and then filtered. From this filtrate two 3 ml. portions were taken, 1 ml. of 3% (w/v) sodium borate was added to one and 1 ml. of homocysteine solution (1.5 mg./ml.) to each. [Homocysteine reduces dehydroascorbic acid (Hughes, 1956) except in the presence of borate.] The two extracts were titrated. The difference, representing total ascorbic acid and probably nothing else, showed 3.34 mg./g.

The results obtained with the hydrazine and homocysteine methods provide evidence that the indophenol-reducing substance extracted from iris leaves is ascorbic acid.

Oxidase. Leaves of many plants, when ground to a paste and left in contact with air, lose ascorbic acid rapidly by oxidation. The phenomenon is well known and the loss is presumed to be enzymic (e.g. Szent-Györgyi, 1931; Butt & Hallaway, 1958). Iris leaves treated in this way lost relatively little ascorbic acid. In one test the control had an ascorbic acid content of 3.81 mg./g. as compared with 3.37 for a paired sample, the pulp of which was kept for 0.5 hr. From 14 other batches of iris leaves, the pulped samples lost varying amounts of

ascorbic acid in 0.5 hr., the average being 12% S.D. $\pm$ 13.4. In one test the loss was 3% in 5 min. and 16% in 1.5 hr. A solution of ascorbic acid (1 mg./ml., comparable with that in diluted leaf juice) in 0.1 M-phosphate buffer, pH 6.5, lost 5% in 0.5 hr., 12% in 1.5 hr. Under similar conditions of testing, pulped leaves of nasturtium (*Tropaeolum majus*) lost 90% of their ascorbic acid in 5 min. From pulped leaves of sugar beet (*Beta vulgaris* cultivar) and of spinach (*Spinacia oleracea*) all the ascorbic acid disappeared in 5 and 15 min. respectively. Leaves of lucerne (*Medicago sativa*) lost 26% in 5 min. and 64% in 0.5 hr. Cabbage and Brussels sprouts (*Brassica oleracea* cultivars) each lost 60% in 0.5 hr.

Tests for inhibitors. The low oxidase activity of iris preparations might be due to an inhibitor acting either directly on the oxidase or indirectly by regenerating ascorbic acid from its oxidized product. Such inhibitors were sought in the following tests.

Of three replicates of iris leaf, *A* was extracted at once; *B* was ground under buffer and left for 1.5 hr.; *C* was treated similarly to *B* but the grinding was done in mM-ethylenediaminetetra-acetic acid. The rates of loss of ascorbic acid in *B* and *C* were both 14% in 1.5 hr., which suggested that the oxidase enzyme had not been inhibited by metal ions in the extraction medium.

An extract of iris leaf was made in 0.1 M-phosphate and 0.4 M-sucrose and dialysed against distilled water for 17 hr. to remove possible inhibitors of low molecular size. Ascorbic acid (0.05% final concentration) was added to part of the extract which was aerated. Only 7% of the ascorbic acid was lost in 6 hr.

Dehydroascorbic acid and reduced glutathione were added to a dialysed extract of iris leaf to give concentrations of 0.01 M for each. After 15 min. none of the dehydroascorbic acid had been reduced anaerobically or aerobically.

The flesh of the cucumber (*Cucumis sativus*) is rich in ascorbic oxidase (Todokoro & Takasugi, 1939). Flesh of an outdoor-grown cucumber harvested in September was comminuted and suspended in its own weight of 0.1 M-phosphate buffer, pH 6.5, and used as source of oxidase. Of duplicate samples (about 1 g.) of iris leaf one was extracted immediately. To the other, 0.1 ml. of the oxidase preparation was added, and after it had been thoroughly ground it was left for 0.5 hr., when the pulp was analysed for ascorbic acid. The difference in ascorbic acid content, representing oxidation, was 71%. In another pair set up with a solution of ascorbic acid instead of iris leaf the corresponding loss was 41%. There was thus no evidence of an inhibitor in iris leaf. When a more active preparation of oxidase was added to iris the ascorbic acid was all lost in 15 min.

DISCUSSION

The value reported here for the iris (4 mg./g. fresh wt. or 31 mg./g. dry wt.) is among the highest so far recorded for ascorbic acid in leaves. Mirimanoff (1938) reported 1.78 mg./g. fresh wt. for green iris leaves, and 2.01 for etiolated leaves; Fujita & Ebihara (1938) found 1.48 in *Iris kaempferi* and 2.93 in *Gladiolus*. These values are all lower than ours, but it is not unusual for low values to be reported when older methods are used. Our values were confirmed with alternative methods of analysis. P. Hagène (personal communication, 1959) found values of up to 25 mg./g. dry wt. in *Iris tingitana*.

Most green leaves that have been tested contain a system that oxidizes ascorbic acid and exhibits strong activity when the leaves are macerated. The iris leaf showed little activity in this respect. Because low enzyme activity might be associated with high ascorbic acid content, as suggested by Barron, Barron & Klemperer (1936), leaves of nasturtium and lucerne, both rich in ascorbic acid, were tested. Since an oxidase was very active in these leaves, this inverse correlation is unlikely to be general. Neither dialysis nor the addition of a chelating agent enhanced the enzymic activity of iris leaf, and ascorbic acid in iris pulp was oxidized when oxidase in cucumber was added. These findings suggest that the activity of the system that oxidizes ascorbic acid is low because the enzyme is only present in small amounts rather than because an inhibitor is also present.

The possibility exists that ascorbic acid is indeed oxidized but immediately reduced by enzyme systems in iris-leaf macerate (Hopkins & Morgan 1936; Crook, 1941). However, there was no evidence for the presence of dehydroascorbic acid reductase, because after dehydroascorbic acid and reduced glutathione were added to an extract of iris leaf no extra ascorbic acid was produced. The further fact that after dialysing an iris extract, which might be expected to remove substrates or coenzyme necessary for the activity of reducing systems, the oxidation rate of added ascorbic acid was still low also makes this possibility seem unlikely.

The low activity of the ascorbic acid-oxidizing system in iris brei is unusual and does not appear to have been reported for any other green leaf.

Note added, February 1960. The iris leaf is also exceptional in that a carotene-oxidizing system is absent (Booth, 1960).

SUMMARY

1. The average ascorbic acid content of leaves of several batches of different varieties of Iridaceae was 4.0 mg./g. fresh wt., or 31 mg./g. dry wt. These are some of the highest concentrations recorded for a leaf.

2. The concentration, based on dry weight, was greatest near the base and least at the apex of a leaf. Based on fresh weight, the concentration was almost invariant along the leaf.

3. The oxidase activity of iris leaf towards ascorbic acid was exceptionally low. No evidence for the presence of an inhibitor was obtained, which suggests that the enzyme is present only in low concentration.

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