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SHORT COMMUNICATION

# AN OBSERVATION ON POSSIBLE PHOTOOXIDATION OF ASCORBIC ACID IN STRAWBERRY LEAVES

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**Abstract**—Photooxidation of ascorbic acid *in vivo* failed under conditions intended to duplicate those used in the work of Mapson and Swain,[3] which was reported as successful.

## INTRODUCTION

PHOTOOXIDATION of ascorbic acid (AA) to dehydroascorbic acid (DHA) *in vivo* has been reported for leaves of strawberry and other plants.[1,2] Preincubation for 5 min at 58° was reported as increasing both the rate and extent of subsequent photooxidation without affecting the dark level of DHA.[2] More recently, phytochrome has been implicated in this process, as evidenced by prevention of photooxidation in low-intensity green light by a preirradiation with far red, the far-red effect being red reversible.[3]

A control of the photooxidation of AA would offer a possible lead to the biochemical mechanism of action of the far-red-absorbing (active) form of phytochrome. Repeated attempts in this laboratory to reproduce the phenomenon, as described by Mapson and Swain, have, however, failed to detect any photooxidation. Because of interest in possible phytochrome action, it was thought useful to report a typical negative result, together with a description of the experimental conditions and method of analysis for DHA. This is done in the hope that possible future uncovering of some unanticipated condition(s) necessary for expression of the phenomenon may result.

## RESULTS

To test the precision of the modified analytical method we used DHA freshly prepared by bromine oxidation of ascorbic acid (Mapson, pers. comm.). Small portions of DHA (0·05 mg/ml in 3·2% metaphosphoric acid) were added to larger volumes of AA at the same concentration so that the total ascorbate concentration remained constant. Because extracts from a typical untreated leaf contained DHA at approximately 2 per cent of the total ascorbate (Table 2), we tested solutions of AA containing from 0 to 5 per cent DHA. The results (Table 1) show that DHA can be detected at concentrations somewhat less than 0·5 per cent

[1] L. W. MAPSON, *Biochem. J.* **85**, 360 (1962).
[2] L. W. MAPSON, *Phytochem.* **3**, 429 (1964).
[3] L. W. MAPSON and T. SWAIN, *Phytochem.* **5**, 829 (1966).

TABLE 1. DETERMINATION OF SMALL AMOUNTS OF DHA ADDED TO SOLUTIONS OF AA AT 0·05 MG/ML. THE DHA WAS PREPARED BY OXIDATION WITH BROMINE. ALL VALUES ARE GIVEN AS PER CENT OF THE TOTAL ASCORBATE

| DHA concentration (added) | DHA concentration (determined) | Confidence limits |
|---|---|---|
| 0 | 0·2 | 0·4 |
| 1 | 1·2 | 0·3 |
| 2 | 2·0 | 0·3 |
| 5 | 5·2 | 0·5 |

of the total ascorbate. The confidence limits are calculated for the smallest significant difference between the two mean indophenol-reducing titres inherent in the analysis, and represent the expected range (95 per cent level) of repeated determinations on the same sample.

The data of Table 2 show failure to effect *in vivo* photooxidation of ascorbate in green light in leaves of *Fragaria vesca*. The 2-min pretreatment at elevated temperatures increases the DHA content of the leaves, but there is no further significant increase upon illumination.

TABLE 2. DHA AND TOTAL ASCORBATE CONTENTS OF LEAVES WITH OR WITHOUT A 5 MIN IRRADIATION WITH GREEN LIGHT AT 100 ERGS $CM^{-2}$ $SEC^{-1}$, WITH OR WITHOUT A 2-MIN PRETREATMENT AT DIFFERENT TEMPERATURES. ALL VALUES ARE GIVEN AS $\mu$G/G LEAF TISSUE

| | Light | | | Dark | | |
|---|---|---|---|---|---|---|
| Temperature pretreatment | DHA | Confidence limits | Total ascorbate | DHA | Confidence limits | Total ascorbate |
| None | 49 | 6 | 2572 | 49 | 5 | 2373 |
| None | 50 | 9 | 2380 | 54 | 6 | 2486 |
| 54·5° | 75 | 11 | 2230 | 93 | 10 | 2213 |
| 56·0° | 155 | 10 | 2480 | 135 | 9 | 2320 |
| 57·7° | 372 | 12 | 2385 | 399 | 14 | 2517 |
| 60·0° | 695 | 10 | 2080 | 695 | 12 | 2210 |
| 62·0° | 2160 | 19 | 2210 | 2000 | 14 | 2100 |

Another method for the estimation of DHA in plant extracts is by formation of the dinitrophenylhydrazone followed by chromatography on alumina columns.[4] This method has the advantage of being a direct estimate as opposed to the indophenol method, which depends on measuring a relatively small difference between two large reducing titres. In an earlier experiment, using leaves of the cultivated strawberry (x *Fragaria ananasas*) and unfiltered cool-white fluorescent light at 1000 ft-c for 10 min, the dinitrophenylhydrazone formation method gave DHA values of $30{\cdot}3 \pm 4$ and $34{\cdot}5 \pm 3$ $\mu$g/g for dark and light leaves, respectively.

## EXPERIMENTAL

### *Plant Material*

Clonal plants of *Fragaria vesca* were pot-grown in a greenhouse (80°/70°F) in natural light with a 3 hr interruption with incandescent light at the middle of the dark period. The experiments reported here were performed between February and May, 1967, after a longer period of experimentation on *F. vesca* and other

[4] L. W. MAPSON, *Biochem. J.* **80**, 459 (1961).

plants. Leaves were harvested in the morning, and 2 g samples were washed in distilled water and blotted with tissue under white fluorescent illumination, then placed in plastic sandwich boxes and stored in the dark at 25° for at least 1 hr before extraction.

*Extraction*

The leaf samples were macerated by blending at high speed for 30 sec in 100 ml ice-cold deoxygenated 3·2 per cent metaphosphoric acid (MPA) in the dark. Nitrogen was continuously run into the blender jar 1·5 min before and during the blending process. The resulting slurry was clarified by suction filtration through Whatman No. 3 filter paper in the dark at 4°. The clarified extract was then immediately analyzed for DHA.

*Analysis*

The DHA was estimated in a modification of the method of Hughes,[5] which is based on the increase in indophenol-reducing power of an extract after treatment with homocysteine at neutral pH.

*Total ascorbate (AA plus DHA).* To 25 ml of clarified extract in a 500 ml erlenmeyer flask was added 2 ml of DL-homocysteine (10 mg/ml) followed by the slow addition, with swirling, of 5 ml of 60 per cent $K_2HPO_4$, which brought the pH to 7·0. Following a 15 min incubation at room temperature, which reduces any DHA present to AA, the mixture was reacidified to pH 2·5 by adding 50 ml 10·72 per cent MPA. Five ml of the reactified mixture was pipetted into a colorimeter tube, which was then placed in the sliding carrier of a Coleman Universal Spectrophotometer, Model 14. Five ml of 2·6-dichlorophenol-indophenol at 0·0445 mg/ml was added with stirring, the sample placed in the light path, and the $OD_{515}$ read at the second deflection of the galvanometer needle, which occurred approximately 13 sec after delivery of the dye. The concentration of the extract and of the indophenol were such as to give OD readings near the middle of the galvanometer scale (optical densities near 0·3), the region of greatest precision.

*AA alone.* A 25 ml aliquot of the filtered extract was treated exactly the same way as for estimation of total ascorbate, except that the order of addition of the $K_2HPO_4$ and 10·72 per cent MPA were transposed so that the mixture was never brought to neutrality in the presence of homocysteine, thereby effectively preventing reduction of any DHA present.[5] Homocysteine was included in both samples because it was found to bleach (reduce) indophenol slightly under the conditions specified. Both extract aliquots were treated at the same time, and the indophenol additions were performed on alternate tubes of the eight replicates of each to eliminate changes that might take place in time.

With freshly prepared solutions of ascorbic acid in MPA, the DHA titre was never found to differ significantly from zero. Small amounts of DHA (freshly prepared by oxidation with bromine) added to portions of extract were 100 per cent recoverable within the limits of sensitivity of the analysis. Stock indophenol solutions were prepared weekly at approximately 4-fold concentration standardized against an ascorbic-acid concentration series and stored in the dark at 4°. Stock MPA was prepared semi-weekly and stored at 4°. The homocysteine solution was prepared immediately before use. Because of the length of the manipulations, usually no more than four extracts were prepared and analyzed in one day.

*Treatment of the Leaf Samples*

Heat treatments were given in the following way: A large-volume, stirred, temperature-controlled water bath was brought to a stable temperature approximately 0·5° above the desired temperature. Enough cold distilled water was then added to lower the temperature about 3° below the desired. When the water bath reached the desired temperature (about 15 min), a prepared leaf sample was placed in a perforated plastic sandwich box. The box was immersed in the water bath and agitated for 2 min in the dark. The sample was then returned to its original container. Subsequent extraction was always carried out within 15 min. Since only one sample at a time was treated (in order that each analysis could be performed immediately after extraction and clarification), the foregoing procedure was necessary because of slow fluctuations in the temperature of the water bath throughout the day.

The light source consisted of two standard cool-white fluorescent lamps positioned immediately beneath 3 cm of 5 per cent acidulated $CuSO_4$. The purpose of the $CuSO_4$ filter was to remove the small amount of far-red light emitted by the source. The green filter, two layers of green gelatin filter[6] contained between sheets of glass, was placed directly above the $CuSO_4$ filter. Leaf samples to be irradiated were placed directly on the green filter, abaxial side down. The intensity at the level of the leaves with both filters in place was approximately 100 ergs $cm^{-2}$ $sec^{-1}$, the illuminance, 40 ft-c. Light treatments were of 5 min duration, since Mapson and Swain reported the photostationary state of DHA content is reached within 2 min after the beginning of illumination with green light at 25 ergs $cm^{-2}$ $sec^{-1}$ [3]. All light treatments were given at 15°. Blending was begun within 10 sec after removing the sample from the light source. Dark controls were treated exactly the same, except that the light was not turned on.

[5] R. E. Hughes, *Biochem. J.* **74**, 203 (1956).
[6] R. B. Withrow and L. Price, *Plant Physiol.* **32**, 244 (1957).