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STUDIES ON THE FORMATION OF ASCORBIC ACID IN DETACHED APPLE LEAVES[1]

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Giroud et al (4) found a close relationship between light intensity, chlorophyll content and ascorbic acid content of green plant tissues, supporting the concept that ascorbic acid formation is directly related to photosynthesis. In chlorophyll-free tissues and in seedlings germinating in the dark, Reid (13) postulated that ascorbic acid was formed by in-dark conversion of a light induced precursor. This hypothesis was supported by Aberg (1) who found that more ascorbic acid accumulated in pea leaves in the dark than in the light, but that the quantity accumulated was related to the light intensity of the previous day. Similarly, Popovskaja (11), and Prokoshew (12) reported the formation in light of a precursor of ascorbic acid in the leaves of potato and tobacco plants which, transported to the tubers and fruits, is converted into ascorbic acid independently of light.

The actual precursor of ascorbic acid has been the subject of much investigation and many substances have been proposed as the precursor. Horowitz and King (6) and Mapson et al (8, 9) have recently presented conclusive evidence of two pathways of ascorbic acid synthesis in which D-glucuronic acid, D-galacturonic acid and their respective gamma lactones would be the active intermediates.

The ascorbic acid content of apples has been determined frequently, but the synthesis of ascorbic acid in apple leaves has received little attention. Because ascorbic acid synthesis in apples may be analogous to the mechanism in potato tubers and tobacco fruits, the present study was undertaken to obtain information on the precursor and the influence of light and darkness on ascorbic acid synthesis in detached apple leaves.

Materials and Methods

The apple leaves used (*Malus sylvestris* Mill., var. McIntosh and Rhode Island Greening) all came from one-year-old shoots on three-year-old root systems. To further increase the probability of obtaining uniform material, these shoots were grown in the greenhouse in quartz sand with nutrient solution applied regularly. When the terminal bud had formed, the shoot was four to five feet in height and bore 30 to 40 leaves. Only the top 20 full-grown leaves were used, each of which weighed between 0.6 and 1.6 gm and had a surface area of 20 to 70 cm^2.

The experimental techniques used by previous workers consisted of either floating whole leaves (1) or discs of tissue (2, 15) or placing germinating seedlings (8) on the desired solution. To detect changes in the ascorbic acid content of the material, resulting from experimental treatments, they determined the ascorbic acid content of representative aliquots of the treated material and water controls at zero time and at certain time intervals thereafter.

In the present study the experimental solutions were fed to detached apple leaves through the petiole. The easily manipulated woody petiole was inserted in a piece of 3 mm glass tubing, slightly tapered at the end to ensure a tight fit. Adhesive tape sealed the petiole in the tubing and completely prevented leakage of the solution at this junction. The other end of the tapered glass tube was inserted into a one inch piece of rubber tubing. With a glass tube, drawn out in a long fine point, some of the desired solution was put in the tapered glass tubing, making sure that no air bubbles were present. Subsequently a 100-ml Midvale bottle was filled with the desired solution until some of the liquid started to pour out of the side arm tube. As soon as this occurred the container unit was tilted over slightly in order to prevent further loss of solution. Holding the container unit in one hand and the leaf, attached to the tapered glass tube and rubber tubing (previously filled with solution) in the other hand, the two parts were put together while making certain that no air bubbles entered the system. This method kept detached apple leaves in a healthy condition for more than three weeks when the solution level in the container was maintained. Preliminary results (3), indicated that backward movement of solutes from the leaf into the Midvale bottle occurred only from autolyzed tissue in direct contact with the water in the container.

In preliminary experiments on sampling technique

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TABLE I

VARIATIONS IN FRESH WEIGHT AND ASCORBIC ACID CONTENT IN 1-CM² DISCS OF APPLE LEAVES FROM A SINGLE TREE

LEAF NO.	DISC NO.	WT OF LEAF DISC	ASCORBIC ACID
		mg	*μgm/disc*
A	1	14.7	87
	2	14.7	86
	3	14.5	87
	4	14.6	86
B	1	13.4	62
	2	13.6	65
	3	13.5	65
C	1	17.1	67
	2	17.0	62
	3	17.2	62
	4	17.0	64
D	1	17.0	80
	2	17.0	92
	3	17.1	88
	4	17.0	94
E	1	16.8	54
	2	17.0	56
	3	17.0	57
F	1	15.1	61
	2	14.9	64
	3	15.1	61
	4	14.9	61

it was found that the ascorbic acid content of whole leaves of one tree and also of comparable leaves of different trees varied considerably. None of these variations could be explained on the basis of age, position on the tree, leaf weight, phyllotaxy or internode length. However, it is shown in table I that variations in ascorbic acid content and fresh weight of 1 cm² discs taken from one leaf are small (about 5 %) as long as care is taken to avoid inclusion of midrib or primary veins. Therefore one to three one-square-centimeter discs were cut from each experimental leaf at the beginning of an experimental run, and additional discs were cut at specified time intervals thereafter. The discs of each sample were taken from both sides of the leaf. The ascorbic acid analyses were performed immediately after each sampling and either the combined discs from each leaf or the combined discs from the leaves receiving the same treatment were analyzed each time as one sample. The results are expressed as micrograms of ascorbic acid per disc because this does not require separate weighing of the discs.

Ascorbic acid, dehydroascorbic acid and 2,3-diketo-l-gulonic acid were determined according to the method of Roe et al (14), using the "Kromatrol" colorimeter with a 525 mμ filter. Because the concentrations of the last two compounds were found to be negligible in apple leaves (3), only the combined values of ascorbic acid, dehydroascorbic acid, and 2,3-diketo-l-gulonic acid are reported.

The equipment used for studies with C^{14} labelled CO_2 consisted of an air tight glass cabinet. The four longitudinal walls (42″ high by 22″) were glass windows, one of which could be conveniently removed to place the required material in the cabinet. The top and bottom of the cabinet were made of ¾-inch plywood. Several openings were made in the top for necessary connection for a thermometer, hygrometer, pressure gauge and $C^{14}O_2$ supply. The $C^{14}O_2$ was produced by mixing a small quantity labelled $BaCO_3$ with excess lactic acid. The calculated CO_2 concentration in the cabinet at the beginning of an experimental run was 0.05 %. After exposure to $C^{14}O_2$ for certain lengths of time leaf discs were analyzed for their ascorbic acid content according to the method of Roe et al (14). Aliquots of the HPO_3 extract were used for preparing the hydrazines which were filtered off on an extra-fine glass sintered plate. The hydrazines were dried on this plate and then put in the Geiger counter (standard windowless flow counter), to determine the counts per minute. The results are expressed as counts per minute per 100 micrograms ascorbic acid and may be denoted as the "specific activity".

EXPERIMENTAL RESULTS

ABSORPTION EXPERIMENTS WITH SUGAR AND SUGAR DERIVATIVES: A number of substances were absorbed by detached apple leaves using 0.1 and 0.5 % concentrations in the Midvale bottle at low (600 ft-c) and high (1700 ft-c) light intensity for 24 and 48 hours. These substances were D-glucose, D-fructose, L-sorbose, sucrose, fructose-1,6-diphosphate, D-sorbitol, D-glucuronic acid and its potassium, calcium and sodium salt, L-gulonolactone, 2-keto-l-gulonic acid and its sodium salt and D-galacturonic acid. Glucosecycloacetoacetic acid and its ethyl ester were also included because, according to Nath et al (10) these serve as precursors of ascorbic acid in *Phaseolus Mungo*.

At high light intensity, sorbose, sucrose, fructose-1,6-diphosphate, D-galacturonic acid, glucosecyclo-

TABLE II

CHANGES IN THE ASCORBIC ACID CONCENTRATION OF DETACHED APPLE LEAVES WHEN VARIOUS SOLUTIONS WERE ABSORBED THROUGH THE PETIOLE AT 1700 FT-C AT ROOM TEMPERATURE

TREATMENT	NO. LEAVES TREATED *	AV INCREASE IN ASCORBIC ACID	
		24 HRS	48 HRS
		μgm/disc.	*μgm/disc*
D-Glucuronolactone, 0.1 %	24	14 **	16
L-Gulonolactone, 0.1 %	18	3	8
D-Galacturonic acid, 0.1 %	12	4	3
Glucose, 0.1 %	12	8	20
Fructose, 0.1 %	18	8	14
H_2O control	36	6	15

* The combined discs from each leaf at each sampling period were analyzed as one sample.

** Significantly different from H_2O control at 1 % level.

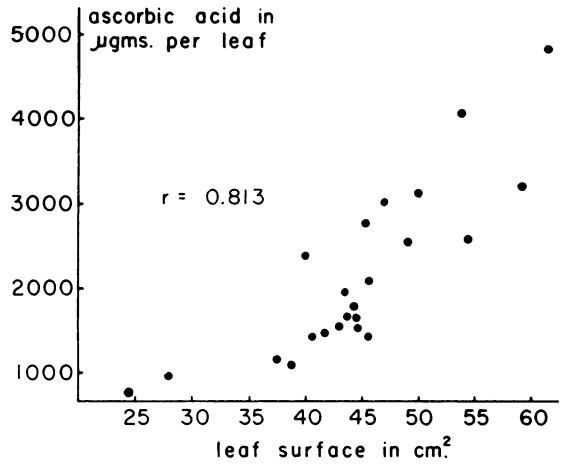

FIG. 1. Relationship between ascorbic acid content of apple leaves and their surface area. All leaves were taken from one shoot.

acetoacetic acid and its ethyl ester, l-gulonic and 2-keto-l-gulonic acids and their sodium salts, and the calcium, potassium and sodium salts of D-glucuronic acid did not increase the ascorbic acid content of apple leaves. Moreover, the 0.1 and 0.5 % solutions of the sodium, calcium and potassium salts of the sugar acids were toxic.

Table II summarizes the experiments with 0.1 % solutions of glucuronolactone, gulonolactone, galacturonic acid, glucose and fructose. Glucuronolactone caused a significant increase in the ascorbic acid concentration after 24 hours, but gulonolactone and D-galacturonic acid, which were proposed as intermediates by Isherwood et al (7) induced less ascorbic acid snythesis than the water control although the decrease was not statistically significant. Similarly the increases in ascorbic acid concentration from glucose and fructose were not significantly greater than the water control.

Mapson et al (9) also found that L-gulonolactone caused no increase in ascorbic acid when they used cytoplasmic particles from pea seeds and cress seedlings. They suggested that the natural path of ascorbic acid synthesis is via the galacturono-galactonolactone rather than by that of glucurono-gulonolactone. However, the results of the experiments with detached apple leaves do not support the galacturono-galactonolactone pathway. Moreover, the negative results obtained with gulonolactone suggest that the active intermediate between glucuronic acid and ascorbic acid may be derivative of the sugar acid other than the gamma lactone.

RELATIONSHIP BETWEEN ASCORBIC ACID CONTENT AND SURFACE AREA OF APPLE LEAVES: In an attempt to indicate a possible relation between the ascorbic acid content of individual whole apple leaves of one tree and also of comparable leaves of different trees with respect to position on the tree, fresh leaf weight, age, phyllotaxy and internode length, a large number of ascorbic acid analyses were performed. The results of these analyses showed that the variations found in the ascorbic acid content of whole apple leaves could not be explained on the basis of age, position on the tree, leaf weight, phyllotaxy or internode length. However, representative date (fig 1) show a high correlation (r = 0.813) between ascorbic acid content of apple leaves and their surface area. This possibly reflects the influence of photosynthesis on ascorbic acid formation and confirms earlier work of Reid (13) with young cowpea plants.

RESULTS OF EXPERIMENTS WITH DIFFERENT LIGHT INTENSITIES: In initial experiments (3) changes in the ascorbic acid and dehydroascorbic acid content of detached apple leaves under low light intensity (600 ft-c) were negligible for periods up to 144 hours. Under exposure to 1700 ft-c a small increase was noticed after 48 hours. The results at low light intensity immediately prompted the suggestion that utilization equalled synthesis.

To determine this, apple leaves were subjected to C^{14} labelled CO_2 at low and high light intensities for a maximum period of six hours. The results are recorded in table III. Although only negligible changes in the ascorbic acid concentration could be detected at both light intensities, and although considerably more labelling occurred at 1700 ft-c the incorporation of C^{14} at 600 ft-c indicates that ascorbic acid was synthesized at the low light intensity. Variations between the different samples are large but are understandable in view of the work of Heinicke (5) who found in leaves from one apple tree, which were well paired as to position on the tree, weight and surface area, that only 12 of the 49 pairs had photosynthetic activity differences of 5 % or less, while 32 pairs had more than 50 %.

TABLE III

SPECIFIC ACTIVITY OF ASCORBIC ACID FROM APPLE LEAVES AFTER LEAVES HAD PHOTOSYNTHESIZED IN AN ATMOSPHERE OF $C^{14}O_2$

SAMPLE * NO.	LIGHT INTENSITY	TIME	CHANGE IN ASCORBIC ACID CONC	SPEC ACTIVITY OF ASCORBIC ACID
	ft-c	*hrs*	*µgm/disc*	*cpm/100 µgm*
1	600	4	−2	39
2	"	4	0	51
3	"	6	2	47
4	"	6	−1	60
5	"	6	−3	60
6	"	6	−2	31
7	1700	3	0	98
8	"	3	−1	51
9	"	4	1	92
10	"	4	3	119
11	"	4	2	133
12	"	4	0	158
13	"	6	2	196
14	"	6	3	131
15	"	6	2	105

* Each sample consisted of 12 discs, three from each of 4 leaves.

TABLE IV

CHANGES IN THE SPECIFIC ACTIVITY OF ASCORBIC ACID IN APPLE LEAVES SUBJECTED TO VARIOUS TREATMENTS OF LIGHT AND DARK, AT ROOM TEMPERATURE IN ORDINARY ATMOSPHERE

SAMPLE NO.*	SPEC ACTIVITY OF ASCORBIC ACID AFTER 6 HRS AT 1700 FT-C	CONDITIONS OF SUBSEQUENT TREATMENT	SPEC ACTIVITY OF ASCORBIC ACID AFTER SUBSEQUENT TREATMENT
	cpm/100 μgm		*cpm/100 μgm*
1	133	3 hrs dark	140
2	158	"	179
3	105	15 hrs dark	155
4	131	"	151
5	67	3 hrs at 600 ft-c	98
6	31	" "	51
7	28	3 hrs at 1700 ft-c	39
8	30	" "	51
9	31	" "	47
10	35	" "	68
11	92	15 hrs at 1700 ft-c	190
12	119	" "	169

* Each sample consisted of 12 discs, three from each of 4 leaves.

Table IV gives data on the specific activity of ascorbic acid in apple leaves directly after removal from a C^{14} labelled carbon dioxide atmosphere and again after these leaves had been subjected to subsequent different light or dark treatments under normal atmospheric conditions. The incorporation of C^{14} into ascorbic acid continues after removal of the external $C^{14}O_2$ supply. Samples 1 to 12 show that this continued labelling occurs in the light as well as in the dark, indicating that only certain steps of ascorbic synthesis in detached apple leaves are light sensitive.

SUMMARY

In the present study, D-glucuronolactone caused a significant increase in ascorbic acid content of detached apple leaves after 24 hours at 1700 ft-c. After 48 hours no significant increase was found with any of the experimental substances including D-glucuronolactone, L-gulonolactone, D-galacturonic acid, glucose and fructose when compared with the water control. The high correlation coefficient between ascorbic acid content and leaf area indicated the close relationship between photosynthetic activity and ascorbic acid content in apple leaves. The light sensitivity of ascorbic acid synthesis was confirmed by $C^{14}O_2$ experiments but the results suggest that only certain steps in this synthesis are light sensitive.

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