Photo-Oxidation of Ascorbic Acid in Leaves

By L. W. MAPSON

Low Temperature Research Station, Downing Street, Cambridge

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These studies were planned as an extension of previous work (Mapson, 1961a) on investigations into the possible role of ascorbic acid and its oxidized forms as electron carriers in plant respiration, and the present investigation was also carried out with this object primarily in view. The results to be described in this paper suggest that the ascorbic acid system may function as an electron-transferring system during photosynthesis, in addition to any such role in respiration.

MATERIALS AND METHODS

Leaves. The leaves used in most of these experiments have been those of the strawberry plant, though several experiments have been conducted with cabbage, privet, poplar, spinach and iris leaves. The leaves were usually collected 17 hr. before the start of an experiment and left for this period in the dark with their petioles in water at 15°. The petiole and midrib were removed from strawberry, privet and poplar leaf samples, and the residual laminae were well mixed and representative samples (1–5 g.) taken for the experiments, which were all conducted at 15°. When iris leaves were used, 1 in. segments of the leaf were prepared and representative samples taken.

Introduction of inhibitors and other substances into the leaf. The compounds used in these studies were supplied to the leaves by immersing the petioles in the various solutions instead of water and allowing the leaves, held in the dark at 15°, to take up the solution for a period of 17 hr. Under these conditions the leaves absorbed about 0.9 ml. of solution/g. of leaf. Assuming no differential absorption the concentration of the compound in the leaf would be expected to be approximately the same as in the solution supplied. To avoid confusion the figures quoted in the text are the concentrations of the solutions actually supplied to the leaves.

Illumination. The light-source chosen was that of a tungsten-filament lamp (150 w), and the degree of illumination of the leaves was varied by altering the distance between lamp and the leaves spread on a flat surface, usually in glass vessels which permitted the atmosphere over the leaves to be changed. In those experiments where leaves were illuminated for more than 1 or 2 min. with light of high intensity, a rapid current of air was passed between the light-source and leaves, to prevent any rise in temperature.

Extraction of the leaves. The conditions for extraction of the leaves (extraction at –3° with oxygen excluded) were those described by Barker & Mapson (1959), which prevent the formation of dehydroascorbic acid during extraction. To arrest the reaction rapidly after the light was removed,

cold metaphosphoric acid (–3°) was poured on to the leaves and the whole mixture immediately blended.

Estimation of L-ascorbic acid and dehydro-L-ascorbic acid. L-Ascorbic acid was estimated in metaphosphoric acid extracts by titration against 2,6-dichlorophenol-indophenol (Barker & Mapson, 1959). At least six titrations for each extract were carried out, and the mean of all titres was determined. Individual titres did not vary from the mean by more than ±1%. In most of the experiments reported in this paper dehydro-L-ascorbic acid was estimated after its reduction to L-ascorbic acid by homocysteine (Hughes, 1956). Reduction of dehydro-L-ascorbic acid was also achieved by the method of Mapson & Ingram (1951); no significant differences were observed in the results obtained by these methods.

These results were generally confirmed by the demonstration that the substance produced on illumination of the leaves behaved like dehydro-L-ascorbic acid in that (1) it readily formed a complex with borate (cf. Huelin, 1949) in its oxidized form but not after reduction by homocysteine, (2) it reacted with 2,4-dinitrophenylhydrazine to yield an osazone that behaved identically, both in its spectrum in 9N-H₂SO₄ and its behaviour on chromatographic analysis (Mapson, 1961b), with the osazone formed from authentic dehydroascorbic acid.

However, the possibility that a substance other than dehydro-L-ascorbic acid was also formed on illumination of the leaf, and was reduced on treatment by homocysteine, thereby being estimated as ascorbic acid, could not be entirely excluded since the results by the indophenol method of assay were always somewhat higher (15–30%) than those obtained by the 2,4-dinitrophenylhydrazine method (Mapson, 1961b). The results recorded here were obtained by the indophenol method of assay, and are expressed in terms of dehydroascorbic acid.

RESULTS

Formation of dehydro-L-ascorbic acid in illuminated leaves

These studies arose as a result of some observations on the changes in the concentration of dehydro-L-ascorbic acid in detached leaves when these were transferred from air to nitrogen in the light. The initial effect of such a change was a rapid fall in the concentration of dehydroascorbic acid in the leaf, which was succeeded by an equally rapid rise to a new steady-state concentration. A typical experiment with strawberry leaves is illustrated in Fig. 1.

When a similar experiment to that described

above was carried out, with the sole exception that the leaf was transferred from air to nitrogen and from light to dark simultaneously, there was a much more rapid fall in the concentration of dehydroascorbic acid to zero (Fig. 2), which in contrast with the previous experiments remained at this value for as long as the experiment was continued (3 hr.). A similar result was obtained when a leaf in air in the light was transferred to the dark; then, however, the dehydroascorbic acid fell more slowly to a low concentration but not completely to zero as in the experiments carried out in nitrogen (Fig. 2).

The results from these preliminary experiments led to the investigation of the effect of light in more detail and the results illustrated in Fig. 3 (a) show that when strawberry leaves, after a period in the dark, were exposed to differing intensities of

illumination from a tungsten-filament lamp, dehydroascorbic acid was produced, the rate of production and the concentration attained in the steady state both increasing with increase in the intensity of illumination. In these experiments the leaves were placed in a stream of carbon dioxide-free air, to render the conditions comparable with the following experiments, in which nitrogen replaced air. No significant differences were found between the results of this and similar experiments in which carbon dioxide was not removed from the air stream. The comparable effect of light on leaves maintained in an atmosphere of nitrogen was also studied. In these experiments the leaves were placed in the dark in a stream of nitrogen gas for 30 min. before illumination. The results (Fig. 3b) show that dehydroascorbic acid was also produced under these conditions, but the rate of production and the concentration attained in the steady state were less than those achieved under aerobic conditions.

The experiments described above were all carried out on strawberry leaves, but an examination of other leaves, including cabbage, poplar, privet, iris, spinach and spinach beet, indicates that, although quantitative differences exist between the different species, essentially similar reactions occurred in response to illumination. The phenomenon is then a general one probably typical of most leaves.

When dehydroascorbic acid is produced in the leaf on illumination there is an equivalent fall in the concentration of ascorbic acid, and on removal of the light these changes are reversed (Table 1). These latter experiments were carried out on privet leaves because the variability in the ascorbic acid contents between different leaves was much lower than in any of the other leaves examined.

Fig. 1. Effect on the concentration of dehydroascorbic acid in strawberry leaves transferred from air to nitrogen in light (5000 lx.).

Fig. 2. Effect on the concentration of dehydroascorbic acid in strawberry leaves when these were transferred ○, from light (5000 lx. for 15 min.) to dark in air, and ●, from air to nitrogen and light to dark simultaneously.

Fig. 3. Effect of intensity of illumination on the rate of production of dehydroascorbic acid and the steady-state concentration attained in strawberry leaves previously held in the dark. (a) Experiments in air; (b) experiments in nitrogen. Figures on the curves refer to illumination (lx.).

Table 1. *Changes in ascorbic acid and dehydroascorbic acid in privet leaves on illumination*

Values in this and succeeding Tables are expressed as $\mu\text{g.g.}^{-1}$ fresh wt. of leaf. 'Total ascorbic acid' indicates ascorbic acid + dehydroascorbic acid.

Expt. no.	Treatment of leaf	Ascorbic acid ($\mu\text{g.g.}^{-1}$)	Total ascorbic acid ($\mu\text{g.g.}^{-1}$)	Dehydro-ascorbic acid ($\mu\text{g.g.}^{-1}$)
1	Dark	1300	1320	20
	Light*	1264	1317	53
2	Dark	724	735	12
	Light	697	747	50
3	Dark	741	757	16
	Light	702	761	59
4	Dark to light	567	620	53
	Dark to light, back to dark	597	616	19
5	Dark	1465	1478	13
	Dark to light	1430	1480	50
	Dark to light, back to dark	1474	1487	13

* Leaves were illuminated for 5 min. with 5000 lx. when transferred from light to dark, and left 30 min. before assay.

Progress of the oxidative reaction. The results of two experiments showing the progress of the reaction with strawberry leaves held in air and nitrogen are illustrated in Fig. 4. The leaves were placed in their respective atmospheres for 30 min. in the dark before being illuminated. In both experiments there was a latent period between the start of illumination and the oxidative response. The duration of this latent period was not significantly greater in the leaves in nitrogen compared with its duration in the leaves in air. The duration of the latent period in both air and nitrogen was, however, affected by the intensity of illumination, decreasing as this was increased. The data illustrated in Fig. 4 also show that the rate of oxidation was greater in the leaves in air compared with those in nitrogen, and the steady-state concentration of dehydroascorbic acid finally achieved was also higher in the former leaves compared with the latter.

Above 5000 lx. the rate of oxidation was too rapid, with the resources available, to measure with any accuracy. The initial rate of the oxidation was determined with up to 5000 lx., from progress curves such as those illustrated in Fig. 4. The relation between light-intensity and rate of oxidation is shown in Fig. 5; there was no evidence of any fall-off in rate: on the contrary, at the higher light-intensities the rate of oxidation increased proportionately with an increase in the light-intensity.

Reducing system. The oxidative changes described appear to be readily reversible when the light is removed. In air a rapid reduction of dehydroascorbic acid occurs and a new steady-state concentration of it (in strawberry leaves about 12–15 $\mu\text{g.g.}^{-1}$) is attained (Fig. 2). In nitrogen the reduction of the oxidized form of the vitamin is even more rapid and then the steady-state concentration attained is zero. A reducing system is thus operating which is independent of light, and, as

Fig. 4. Formation of dehydroascorbic acid in strawberry leaves on illumination (figures above arrows give intensity in lx.): (a) in air (●) and nitrogen (○); (b) in air.

Fig. 5. Relation between intensity of illumination and the rate of production of dehydroascorbic acid in strawberry leaves.

might be expected, is much more efficient in the absence of oxygen. A further important difference from the photochemical oxidation is that there is an apparent delay between the onset of reduction of dehydroascorbic acid and the cutting off of the light, which suggests that the system is normally functioning under both light and dark conditions. What is not certain from these experiments is whether the electron transfer over this system is more or less rapid in illuminated leaves compared with its activity in leaves in the dark. Some evidence on this point will be discussed below.

Steady-state concentration. The steady-state concentration of dehydroascorbic acid attained in the light is evidently related to the intensity of illumination and to the partial pressure of oxygen in the external atmosphere. A possible explanation is that the steady-state concentration represents the difference in rates of the systems oxidizing ascorbic acid and those reducing the oxidized forms. Mapson (1961*a*) has already produced evidence that a similar equilibrium exists between such oxidizing and reducing systems in other plant tissues. The observations showing that the efficiency of the reducing system is greater in nitrogen than in air are in agreement with this concept, and with the lower steady-state concentration of dehydroascorbic acid obtainable under anaerobic as compared with aerobic conditions with identical conditions of illumination. Further experiments were therefore carried out in an attempt to determine whether the steady-state concentration in the leaf represented a true equilibrium.

The results illustrated in Fig. 6 show that decreasing the light from 5000 lx. to a lower light-intensity, or illuminating darkened leaves with various light-intensities, produces a steady-state concentration of dehydroascorbic acid that is dependent solely on the illumination, and is independent of whether this state is produced by increasing or decreasing the intensity of the illumination. Such results are consistent with the concept of an equilibrium between a photochemical system oxidizing ascorbic acid and a system concerned with the reduction of the oxidized products.

Some comment on our earlier observations is appropriate at this stage. Although an increase of light from 2500 to 5000 lx. increased the rate of the oxidative process by a factor of nearly 4, the steady-state concentration of dehydroascorbic acid was only slightly increased. This relationship is more clearly shown in Fig. 7, where the initial rates of oxidation on the illumination of darkened leaves with different light-intensities are plotted against the concentrations of dehydroascorbic acid finally attained in the steady state. Clearly the steady-state concentration cannot be explained simply on the basis that the concentration of the photo-

chemical oxidant, as the illumination is increased from zero to 5000 lx., reaches a limiting concentration, because the data illustrated in Fig. 5 show that there is no evidence of any fall-off in the rate of oxidation as the light-intensity is increased.

On the questionable assumption that light has no influence on the activity of the reducing system, the maximum rate of reduction of dehydroascorbic acid observed in the dark is still considerably less than the rates of oxidation observed on illumination. Thus in leaves in nitrogen after removal of light (5000 lx.) the observed rate of reduction was $150 \mu\text{g. g.}^{-1} \text{ min.}^{-1}$ and in air about one-third of this, $50 \mu\text{g. g.}^{-1} \text{ min.}^{-1}$. This should be contrasted with the observed rates of oxidation on illumination with light of 5000 lx., which were found to be $300 \mu\text{g. g.}^{-1} \text{ min.}^{-1}$ and $700 \mu\text{g. g.}^{-1} \text{ min.}^{-1}$ in nitrogen and air respectively. If all the ascorbic acid in the leaf ($3000 \mu\text{g. g.}^{-1}$) were available to such a system one might expect that the equilibrium would be stabilized at a much higher concentration of dehydroascorbic acid than in fact it is.

This phenomenon might be explained either by the fact that the rate of oxidation of ascorbic acid

Fig. 6. Changes in the steady-state concentration of dehydroascorbic acid in leaves with changes in illumination. Leaves illuminated with: ●, 5000 lx.; ○, 1000 lx.; △, 500 lx. Changes in illumination from 5000 lx. to either 1000 or 500 lx. are indicated on the graph.

Fig. 7. Relation between rate of production and the steady-state concentration of dehydroascorbic acid in illuminated strawberry leaves.

in the steady state is limited at the higher light-intensities by the actual concentration of ascorbic acid available at the site of the reaction, or it could equally well be explained if the reducing system was likewise stimulated by light and which by its enhanced activity would prevent a marked rise in the steady-state concentration as the illumination increased. The correct explanation is not unimportant because at the steady-state concentration on the first hypothesis the turnover rate might be expected to be relatively small, being limited by the actual concentration of ascorbic acid available for oxidation, whereas on the second hypothesis it might be expected to be much larger. One factor that may also contribute to the activity of the reducing system is the actual concentration of the oxidized form, for, as Fig. 2 shows, the rate of reduction falls as the concentration of dehydroascorbic acid decreases. At low light-intensities this is low and may limit the rate of reduction (cf. Mapson, 1961a); at higher light-intensities the higher concentration of dehydroascorbic acid in the steady state will favour the reduction process.

Influence of ascorbic acid content on the steady-state concentration of dehydroascorbic acid

If the rate of oxidation of ascorbic acid on illumination is limited solely by the ascorbic acid concentration available at the site of the reaction, then this localized concentration might be raised by increasing generally the concentration of the vitamin in the cytoplasm. When the petioles of leaves were immersed in a solution of 1% (w/v) ascorbic acid for a period of 17 hr. before the start of the experiments the ascorbic acid content of a leaf was often increased by a factor of three to four, yet despite this increase no significant effect in the steady-state concentration of dehydroascorbic acid produced on illumination was observed (Table 2). These results are consistent with another fact observed in this work, namely that although the concentration of ascorbic acid in strawberry leaves naturally varied from 1.7 to 7.0 mg. g.⁻¹ of fresh leaf the steady-state concentration of dehydro-

ascorbic acid produced under the same conditions of illumination was constant and did not vary more than 1–2% in leaves taken at the same period in the year. No relation has therefore been found between the concentration of ascorbic acid in the leaf and steady-state concentration of dehydroascorbic acid produced on illumination.

The results of such experiments are, however, open to different interpretations. It may be argued that an increase in the general concentration of ascorbic acid in the cytoplasm may not lead to an increase of ascorbic acid at the actual site of the reaction. However, the chloroplasts (see Discussion) are probably the site of the oxidative reactions studied here, and the evidence available from the present studies indicates that the chloroplast is freely permeable to ascorbic acid; certainly the chloroplasts of most leaves, e.g. iris and privet, lose their ascorbic acid and dehydroascorbic acid readily on washing. With these leaves two washings are sufficient to decrease their total ascorbic acid content (ascorbic acid + dehydroascorbic acid) to zero. With the strawberry leaf the chloroplasts are less permeable but loss does occur here also on washing. Such evidence suggests that the chloroplast is permeable to ascorbic acid and there is no reason to suppose that a general increase in the ascorbic acid of the cytoplasm would not be reflected in a higher concentration in the chloroplasts themselves.

Production of dehydroascorbic acid in leaves poisoned with cyanide

The results described above could be interpreted equally well on the basis that the steady-state concentration of dehydroascorbic acid is the result of an equilibrium between two opposing sets of reactions, those concerned with oxidation and those with reduction, only if the assumption is also made that light stimulates reduction as well as oxidation.

Even in the dark in air there is a small but steady concentration of dehydroascorbic acid maintained in the leaf. In strawberry leaves this averages

Table 2. *Relation between the concentration of ascorbic acid and the steady-state concentration of dehydroascorbic acid in illuminated leaves*

Leaves were illuminated for 15 min. at 5000 lx.			
Expt.	Pretreatment	Ascorbic acid ($\mu\text{g. g.}^{-1}$)	Dehydro-ascorbic acid ($\mu\text{g. g.}^{-1}$)
Strawberry leaves (14. viii. 61)	In water, 17 hr.	3215	130
	In ascorbic acid (1%, w/v), 17 hr.	8413	130
Poplar leaves (28. ix. 61)	In water, 18 hr.	1794	121
	In ascorbic acid (1%, w/v), 18 hr.	7935	125
Privet leaves (9. ii. 62)	In water, 17 hr.	1020	70
	In ascorbic acid (1%, w/v), 17 hr.	1623	67

13 $\mu\text{g. g.}^{-1}$. In leaves poisoned with cyanide an alteration in this steady-state concentration occurs, the extent of the change depending on the concentration of cyanide used. Cyanide in these experiments was administered to the intact leaf by feeding via the petiole for a period of 17 hr. before test. The results (Fig. 8) show that as the concentration of cyanide is increased there is first an increase in the steady-state concentration, which is more than doubled at a concentration of cyanide of approximately $10 \mu\text{M}$ but decreases below that present in unpoisoned leaves to near zero as the concentration of cyanide is still further raised. These results are reminiscent of the many reports in the literature of the stimulative effect of cyanide in low concentrations, and its inhibitory effect at high concentrations on respiration: Warburg (1919), Barker & Hanes (1931), Marsh & Goddard (1939) and Macdonald & De Kock (1958). Preliminary results show that cyanide in low concentrations does indeed increase the oxygen uptake of darkened strawberry leaves; these results will be reported later.

The chief point of interest in the present paper is the contrast between the effects of cyanide described above and its effect on the leaves when these are illuminated. The results of such experiments (Fig. 8) show that, with cyanide at all concentrations above $0.1 \mu\text{M}$, the steady-state concentration in the leaves falls to zero when the leaves are illuminated. There is thus not only a marked inhibition of the photochemical oxidation in the cyanide-poisoned leaves, but an actual fall in the steady-state concentration of dehydroascorbic acid below that in normal darkened leaves. Similar results were obtained with azide.

These results are explicable on the assumption that light stimulates the reduction of dehydroascorbic acid, even in cyanide-poisoned leaves, for otherwise it is difficult to explain the complete absence of any dehydroascorbic acid in these leaves in the light despite the fact that in the dark at certain concentrations of cyanide the steady-state concentration of dehydroascorbic acid has been increased. The underlying assumption in this argument is that the normal dark reactions are also operating in the light, and there seems no reason to doubt this.

An attempt has been made to obtain direct evidence that the rate of reduction of dehydroascorbic acid is stimulated by light. For this purpose the rate of reduction of dehydroascorbic acid, measured by following the progress of the reaction in cyanide-poisoned leaves when illuminated, was compared with the rate observed in normal leaves containing an equal concentration of dehydroascorbic acid. This was achieved by exposing non-poisoned leaves to a light-intensity

(800 lx.) that produced a steady-state concentration of dehydroascorbic acid of approximately $30 \mu\text{g. g.}^{-1}$, i.e. the same concentration obtained with darkened leaves poisoned with cyanide ($50 \mu\text{M}$). The initial rates of reaction determined from the progress curves (Fig. 9) was much greater in illuminated cyanide-poisoned leaves compared with the rate in non-poisoned leaves on removal of light (Table 3). The reduction process stimulated by light in the cyanide-poisoned leaves showed the

Fig. 8. Effect of cyanide on the steady-state concentration of dehydroascorbic acid in strawberry leaves in the dark and light. ○, Illuminated leaves (5000 lx.); Δ, darkened leaves.

Fig. 9. Effect of light on reduction of dehydroascorbic acid in cyanide-poisoned leaves. Δ, ○, ×, ●, Cyanide-poisoned leaves illuminated at zero time (intensities in lx. are shown on curves); □, normal leaves transferred from light (500 lx.) to dark at zero time.

same characteristics as the photo-oxidation of ascorbic acid in normal leaves, i.e. (1) a latent period occurred between onset of illumination and reduction, (2) the duration of this latent period decreased with increase in illumination and (3) the rate of the ensuing reaction also increased with an increase in the illumination. With light of 10 000 lx. the rate was so rapid that only an approximate estimate was possible.

If cyanide (50 μM) completely suppresses photo-oxidation without any effect on photoreduction, the results (Table 3) indicate that with an illumination of 5000 lx. the rate of reduction of dehydroascorbic acid is increased by a factor of 10 over the rate of the reaction in darkened normal leaves. Earlier experiments (Fig. 2) showed that the rate of reduction in darkened leaves in air was equivalent to 50 $\mu\text{g. g.}^{-1} \text{ min.}^{-1}$, a rate that might be expected to increase to 500 $\mu\text{g. g.}^{-1} \text{ min.}^{-1}$ with light of 5000 lx. With the same light-intensity the estimated rate of photo-oxidation of ascorbic acid was approximately 700 $\mu\text{g. g.}^{-1} \text{ min.}^{-1}$.

In the present experiments the rate of photo-oxidation of ascorbic acid has been determined on the same batch of leaves used for the experiments described in Table 3 and thus it is possible to make a more direct comparison between rates of photo-oxidation and photoreduction. The rate of oxidation was equivalent to a turnover of 240 $\mu\text{g. g.}^{-1} \text{ min.}^{-1}$ at 5000 lx. and produced a steady-state concentration of 65 $\mu\text{g. g.}^{-1}$. As against this the rate of reduction of dehydroascorbic acid in cyanide-poisoned leaves was equivalent to 150 $\mu\text{g. g.}^{-1} \text{ min.}^{-1}$, starting from a steady-state concentration of dehydroascorbic acid of 30 $\mu\text{g. g.}^{-1}$ (Table 3) at the same light-intensity. This rate might be expected to be increased if the steady-state concentration had been 65 $\mu\text{g. g.}^{-1}$. Such estimates of the rates of these opposing reactions suggest that the steady-state concentration of dehydroascorbic acid represents an equilibrium between the two reactions.

The rate of photo-oxidation and the steady-state concentration attained in these later experiment was less than those observed in the earlier experiments (Fig. 3). This difference appears to be

due to the age of the leaf; in the earlier experiments young summer and autumn leaves were used; in the later experiments older leaves picked in the winter months were used. For a given intensity of illumination the steady-state concentration of dehydroascorbic acid tends to fall as the leaf ages.

These results therefore afford direct evidence that the reduction of dehydroascorbic acid, like the oxidation of ascorbic acid, is stimulated by light and suggest that both photo-oxidation and photo-reduction are initiated by a photochemical reaction common to both.

Effect of 1'-(3,4-dichlorophenyl)-1,1-dimethylurea, hydroxylamine and o-phenanthroline

The photochemical nature of the oxidation of ascorbic acid naturally raised the question whether the oxidant primarily responsible was the so-called photohydroxyls produced by the photolysis of water in a Hill reaction (Hill & Scarisbrick, 1940). Such reactions are all inhibited by hydroxylamine (Gaffron, 1942), o-phenanthroline or 1'-(3,4-dichlorophenyl)-1,1-dimethylurea (Bishop, 1958), the last-named compound inhibiting the photolysis of water at concentrations that are claimed not to influence other major metabolic reactions.

The ability of these three compounds to inhibit the photochemical oxidation of ascorbic acid has been examined. The inhibitor was supplied to the leaf via the petiole under the conditions used to supply cyanide. The results (Table 4) show that all three compounds were effective in inhibiting the oxidation of ascorbic acid in illuminated leaves. The most effective was 1'-(3,4-dichlorophenyl)-1,1-dimethylurea, which produced almost complete inhibition of the reaction when supplied in 0.01 μM solution. The other inhibitors had to be used in higher concentrations to produce complete inhibition; this finding is in agreement with the efficiencies of these substances as inhibitors of the Hill reaction (Bishop, 1958).

One other difference between these inhibitors and cyanide is noteworthy. Although the former compounds inhibit the photo-oxidation of ascorbic acid they do not reduce the steady-state concentration of dehydroascorbic acid to zero, but only to

Table 3. *Effect of light on rate of reduction of dehydroascorbic acid in cyanide-poisoned strawberry leaves*

	Steady-state concn. of dehydroascorbic acid ($\mu\text{g. g.}^{-1}$)	Change	Rate of reduction of dehydroascorbic acid ($\mu\text{g. g.}^{-1} \text{ min.}^{-1}$)
Normal leaves in light (800 lx.)	32	Light to dark	16
Leaves poisoned with cyanide (50 μM) in dark	30	Dark to 1000 lx.	40
	30	Dark to 2500 lx.	60
	30	Dark to 5000 lx.	150
	30	Dark to 10 000 lx.	≈ 400

Table 4. *Inhibitory effect of 1'-(3,4-dichlorophenyl)-1,1-dimethylurea, hydroxylamine and o-phenanthroline on the photo-oxidation of ascorbic acid*

DCMU, 1'-(3,4-Dichlorophenyl)-1,1-dimethylurea.

Expt.	Pretreatment (for 17 hr.)	Illumination	Ascorbic acid ($\mu\text{g. g.}^{-1}$)	Dehydro- ascorbic acid ($\mu\text{g. g.}^{-1}$)
Strawberry leaves	In water	Dark	2440	14
	In water	5000 lx.; 5 min.	2180	86
	In DCMU (1 μM)	5000 lx.; 5 min.	2278	15
	In DCMU (1 μM)	10 000 lx.; 5 min.	2442	16
	In DCMU (1 μM)	Dark	2440	14
Poplar leaves	In water	Dark	1990	12
	In water	5000 lx.; 5 min.	1794	121
	In DCMU (1 μM)	5000 lx.; 5 min.	2616	17
Strawberry leaves	In water	Dark	2758	14
	In water	5000 lx.; 5 min.	4416	105
	In DCMU (0.01 μM)	5000 lx.; 5 min.	3020	21
Strawberry leaves	In water	Dark	4370	10
	In water	5000 lx.; 5 min.	3360	100
	In o-phenanthroline (0.5 mM)	5000 lx.; 5 min.	3120	9
	In hydroxylamine (1 mM)	5000 lx.; 5 min.	2584	28

that existing in darkened leaves. This is in conformity with the fact that they inhibit the initial stages in the photosynthetic process and thereby inhibit both oxidation and reduction reactions resulting therefrom. Cyanide, as shown, appears to have an inhibitory effect on the photo-oxidation reaction without a corresponding inhibitory influence on the reactions associated with reduction.

DISCUSSION

Arnon and his co-workers (Arnon, Whatley & Allen, 1954; Whatley, Allen & Arnon, 1956) first suggested that ascorbic acid acts as an electron carrier in the oxidative chain in photosynthetic phosphorylation. The photo-oxidation of ascorbic acid by isolated chloroplasts has been reported by a number of investigators (Mehler, 1951; Vernon & Kamen, 1954; Wessels, 1954), and Habermann & Vernon (1958) showed that the oxidation of ascorbic acid in air by chloroplasts was mediated by 2,6-dichlorophenol-indophenol.

Marré, Arrigoni & Rossi (1959) found that whereas whole chloroplasts only feebly photo-oxidized ascorbic acid, disrupted chloroplasts readily oxidized it, even in the absence of oxygen, if a hydrogen-acceptor system containing NADP was present.

Mitsui & Ohta (1961) described not only the photo-oxidation of ascorbic acid by illuminated chloroplasts but also obtained some evidence that their preparations would reduce what they termed an endogenous precursor to L-ascorbic acid. This endogenous precursor was not, however, identified. The rates of reaction induced under their conditions were, however, very slow compared with the rates of reaction studied in this paper. Mitsui & Ōi

(1961a) extended their studies to show that in a mixture of spinach chloroplasts and a freshly prepared cytoplasmic fraction, when incubated under anaerobic conditions in the light, a reducing substance was formed that was identified by its spectrum as ascorbic acid. Marré & Arrigoni (1958) found that the oxidation of ascorbic acid in the presence of chloroplasts and an oxidizing agent such as Cu^{2+} ion was retarded by illumination and considered the effect to be due to the reduction of monodehydroascorbic acid by electrons produced by the photolysis of water. Mitsui & Ōi (1961b) found a parallelism between photochemical trans-phosphorylation, photosynthetic phosphorylation, the Hill reaction and the light-induced formation of ascorbic acid in spinach leaves during different stages of development of the leaf.

These studies collectively suggested the possibility that ascorbic acid may play some part in the photosynthetic process. On the other hand, ascorbic acid is so readily oxidized by a number of systems in plant tissues that the observation that it may be oxidized or the oxidized forms reduced by isolated chloroplasts by no means proves that such reactions are taking place *in vivo*. It is noteworthy that in the experiments of Mitsui & Ohta (1961) no photochemical oxidation occurred under anaerobic conditions, and in those of Mitsui & Ōi (1961a) anaerobic conditions were required for the formation of ascorbic acid. However, Vernon & Zaugg (1960) reported that ascorbic acid is photo-oxidized by illuminated chloroplasts even under anaerobic conditions, especially if indophenol dye is also present.

In this work the oxidized form of ascorbic acid actually estimated was dehydroascorbic acid, the first stable product of the oxidation; it seems

probable that *in vivo* the real electron acceptor is the unstable semiquinone form monodehydroascorbic acid, which under our conditions of extraction and assay would be estimated, possibly after dismutation, as dehydroascorbic acid.

The results reported in this paper point to the ascorbic acid system as an electron carrier during photosynthesis in the intact leaf. Moreover the results suggest that the stimulation of both oxidation of ascorbic acid and reduction of the oxidized form, which is initiated in leaves on illumination, is superimposed on oxidation and reduction reactions existing in the tissues in the complete absence of light.

It has been shown that light stimulates the reduction of the oxidized form, only by suppressing the photo-oxidation of ascorbic acid by poisoning the leaves with cyanide. It is difficult to ascertain the quantitative effect of light on these reactions in normal leaves because photo-oxidation of ascorbic acid may not be completely suppressed by cyanide and photoreduction may not be entirely insensitive to cyanide.

Photo-oxidation of ascorbic acid in the steady state may be limited by its concentration at the site of the reaction. Our results are not conclusive on this point; some limitation of the photo-oxidation may occur as a result of this, although if this were the principal factor concerned it is difficult to understand why no relation exists between the concentration of ascorbic acid in the leaf and the steady-state concentration of dehydroascorbic acid produced on illumination. Our results seem to be more explicable on the assumption that the steady-state concentration of dehydroascorbic acid is the result of an equilibrium between photo-oxidation and photoreduction. If this is so then the electron transfer over the ascorbic acid system may be quite considerable.

The significance of our results in relation to the photosynthetic mechanism remains to be determined. One possibility is that ascorbic acid may act as an electron donor, and dehydroascorbic acid, or more probably the intermediate form monodehydroascorbic acid, as electron acceptors, positioned as the intermediate A in the sequence of reactions postulated by Losada, Whatley & Arnon (1961) for non-cyclic phosphorylation in green leaves. If this were so then the complete inhibition of photosynthesis by 1'-(3,4-dichlorophenyl)-1,1-dimethylurea, hydroxylamine or *o*-phenanthroline would be expected to produce a steady-state concentration of dehydroascorbic acid, identical with that existing in darkened leaves, whereas if cyanide inhibited some step in the reaction sequence subsequent to the participation of ascorbic acid, then on illumination the flow of electrons to the oxidized form of ascorbic acid would proceed,

temporarily at least, whereas the flow of electrons from ascorbic acid would be blocked, or partially blocked, with the consequent disappearance of the oxidized form.

SUMMARY

1. Ascorbic acid is rapidly oxidized to dehydroascorbic acid in strawberry leaves when these are illuminated. The oxidation, the rate of which increases with increase in the illumination, occurs under both aerobic and anaerobic conditions. The phenomenon has been observed with leaves of cabbage, iris, poplar, spinach and privet.

2. On removal of light the process is reversed: dehydroascorbic acid decreases to the steady-state concentration observed in darkened leaves. The rate of the reduction is more rapid in nitrogen than in air.

3. The photo-oxidation of ascorbic acid in light leads to an increase in the steady-state concentration of dehydroascorbic acid in the leaf, the extent of this increase depending on the intensity of illumination. The steady-state concentration may be increased by increasing, or decreased by decreasing, the illumination.

4. Evidence that light also stimulates the reduction of dehydroascorbic acid has been obtained from cyanide-poisoned leaves. Cyanide in low concentrations appears to suppress the photo-oxidation of ascorbic without inhibiting that of photoreduction.

5. The steady-state concentration of dehydroascorbic acid in illuminated leaves is explicable on the basis of an equilibrium between photo-oxidation and photoreduction reactions.

6. No relation was found between the concentration of ascorbic acid in the leaf and the steady-state concentration of dehydroascorbic acid produced on illumination.

7. Inhibitors of the photosynthetic process, 1'-(3,4-dichlorophenyl)-1,1-dimethylurea, hydroxylamine and *o*-phenanthroline, suppress both photo-oxidation of ascorbic acid and photoreduction of dehydroascorbic acid, and as a result the steady-state concentration of dehydroascorbic acid in the illuminated leaves is identical with that found in darkened leaves.

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The Nature of Compounds Present in Mixtures of Oxidized and Reduced Flavin Mononucleotides

BY Q. H. GIBSON, V. MASSEY AND N. M. ATHERTON

Departments of Biochemistry and Chemistry, University of Sheffield

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Much recent work on flavin-containing enzymes has followed from the observations of Beinert (1956) on the spectral changes in solutions of reduced flavin mononucleotide during oxidation by molecular oxygen. In addition to the bands due to the oxidized and reduced flavins, bands due to two other compounds were seen, one with maximal extinction near 570 m μ and another with a peak at about 900 m μ (the exact position of the band varied with pH). As the oxidation of FMNH₂ progressed the two new bands increased in intensity to a maximum at about half oxidation and faded again as oxidation was completed. On the basis of concentration and temperature effects the 570 m μ band was attributed to the half-reduced (semiquinoid or free radical) form of the flavin and the 900 m μ band to the dimer of the semiquinone. The conclusion that a free radical species is present in mixtures of FMNH₂ and FMN has been confirmed by electron-spin-resonance spectrometry (Beinert & Sands, 1961), though no quantitative studies were made to relate spectroscopic appearances and electron-spin-resonance signals to the progress of the oxidation.

Massey & Palmer (1962) recently studied the spectra of the enzyme lipoyl dehydrogenase and of model systems and have concluded that both free and enzyme-bound flavin form charge-transfer complexes with oxidized nicotinamide nucleotides and their analogues. These complexes have extinc-

tion bands sufficiently similar to the band at 900 m μ seen by Beinert (1956) to suggest that the 900 m μ band might be due to a charge-transfer complex between FMN and FMNH₂ rather than to a dimer of the semiquinone. Further, if Beinert (1956) was correct in assigning the band at 570 m μ to a flavin semiquinone and the band at 900 m μ to its dimer, then the extinction at 900 m μ should be proportional to the square of the extinction at 570 m μ . Although Gibson & Hastings (1962) have found that this relation is roughly followed in dilute (< 0.5 mM) mixtures of FMN and FMNH₂, Massey & Palmer (1962) observed gross departures from it in more concentrated solutions.

In view of these difficulties and because of the wide use of spectroscopic findings on the model system FMN-FMNH₂ in interpreting the extinction bands found with flavoprotein enzymes, we have extended Beinert's (1956) work, collecting and correlating quantitative results by spectrophotometric, fluorescence and electron-spin-resonance methods.

MATERIALS AND METHODS

Chemicals. FMN was purchased either from The Sigma Chemical Co., St Louis, Mo., U.S.A., or from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Dihydrolipoamide was prepared by the method of Reed, Koike, Levitch & Leach (1959). Sodium dithionite was British Drug Houses Ltd. laboratory reagent; the other chemicals