LIGHT DEPENDENT OXYGEN METABOLISM OF CHLOROPLAST PREPARATIONS. III. PHOTOOXIDATION OF ASCORBIC ACID[1,2]

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Ascorbic acid is a normal constituent not only of the chloroplasts but also of the other protoplasmic components of the cells of green leaves. In spite of its known widespread occurrence in plants, few functions of ascorbic acid in plant metabolism have been established (see review by Mapson, 15). The participation of ascorbic acid as an intermediate hydrogen carrier in photosynthesis has been suggested (16, 17) and certainly the experiments of Krasnovsky on the photochemical reduction of chlorophyll in vitro by ascorbic acid (13, 14) have added new interest to these suggestions. Ascorbic acid was at one time considered to be a necessary component of the photosynthetic phosphorylation system but more recently it has been regarded as having a protective role in preventing inactivation of essential components of the chloroplasts (Arnon, 1).

Soon after he discovered that chloroplasts could use oxygen as a Hill reagent, Mehler (18, 19) reported that rates of net oxygen uptake in the light by reaction mixtures containing chloroplasts, ethanol, and catalase were stimulated several fold if the chloroplasts had first reduced quinone. To determine if it was necessary for chloroplasts to reduce quinone in the light, Mehler added ascorbic acid to the reaction mixture to reduce quinone in the dark. When an excess of ascorbic acid was added, an extremely rapid oxygen consumption occurred on illumination. The nature of this rapid uptake in the presence of ascorbic acid was of particular interest because of the apparent increase in the efficiency of utilization of light energy.

Later, Vernon and Kamen compared the photooxidations of various redox couples by spinach leaf homogenates and sonic extracts of *Rhodospirillum rubrum* (21). The most active couple which they found was the combination of 2,6-dichlorophenolindophenol (DPIP, acting as an electron carrier) and ascorbic acid (acting as the ultimate electron donor). In both plant and bacterial extracts, oxygen uptake in the light continued until oxygen equivalent to the excess ascorbic acid in the system had been consumed. The nature of this rapid DPIP-mediated photooxidation of ascorbic acid has since been investigated using tracer oxygen techniques (Habermann & Vernon, 10). The results of these studies support the hypothesis that ascorbic acid can be oxidized by the oxidized product of photolysis.

This paper describes investigations of the mechanism of the photooxidation of ascorbic acid mediated by chloroplast reaction systems.[4] Addition of ascorbic acid during either a Mehler or exchange reaction results in an immediate acceleration of the rate of net oxygen uptake. Just as the rates of the Mehler and exchange reactions are accelerated after a chloroplast preparation has reduced quinone (19, 8), previous reduction of quinone has a stimulating effect on rates of subsequent ascorbic acid oxidation by such chloroplast preparations. The use of tracer oxygen (which makes possible the simultaneous measurement of rates of oxygen consumption & production) indicated that increases in rates of net oxygen uptake during the oxidation of ascorbic acid are *not* a consequence of an additional oxygen-consuming reaction being super-

Hill reaction (production only):

$$\begin{aligned} 4H_2O &\longrightarrow 4(H) + 4(OH) \\ 2Q + 4(H) &\longrightarrow 2QH_2 \\ 4(OH) &\longrightarrow 2H_2O + O_2 \\ \hline 2H_2O + 2Q &\longrightarrow 2QH_2 + O_2 \end{aligned}$$

Exchange reaction (no net change in oxygen tension, i.e., production equal to consumption):

$$\begin{aligned} 4H_2O &\longrightarrow 4(H) + 4(OH) \\ 2O_2^* + 4(H) &\longrightarrow 2H_2O_2^* \\ 4(OH) &\longrightarrow 2H_2O + O_2 \\ 2H_2O_2^* &\longrightarrow 2H_2O^* + O_2^* \\ \hline 2H_2O + O_2^* &\longrightarrow O_2 + 2H_2O^* \end{aligned}$$

Mehler reaction (production less than consumption of oxygen):

$$\begin{aligned} 4H_2O &\longrightarrow 4(H) + 4(OH) \\ 2O_2^* + 4(H) &\longrightarrow 2H_2O_2^* \\ 4(OH) &\longrightarrow 2H_2O + O_2 \\ 2H_2^*O_2^* + 2CH_3CH_2OH &\longrightarrow 4H_2O^* + 2CH_3CHO \\ \hline 2H_2O + 2\,CH_3CH_2OH + 2O_2^* &\\ \rightarrow 2\,CH_3CHO + 4H_2O^* + O_2 & \end{aligned}$$

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[2] The contents of this paper are part of a thesis submitted to the graduate faculty of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree.

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[4] These reactions can be defined operationally in terms of oxygen metabolism according to the following equations:

imposed on the normal pattern of oxygen metabolism of these chloroplast reactions, but that ascorbic acid is oxidized by reaction with either the oxidized product of photolysis or an intermediate in the pathway to oxygen production. Preliminary reports of these findings were presented at the A.I.B.S. meetings in East Lansing, Michigan, and at the Gatlinburg conference on photosynthesis (7, 9).

Materials & Methods

Chloroplasts were prepared from mature leaves of *Phytolacca americana* L. (pokeweed) according to procedures previously described (Habermann, 8). All reactions were run in conventional rectangular Warburg vessels. Light from a bank of fluorescent bulbs mounted vertically behind the back window of a constant temperature water bath was reflected onto the bottoms of the experimental vessels by a mirror mounted at a 45° angle. Manometric experiments were run at 18.8° C, tracer experiments at 23° C. Adaptation of the mass spectrometer (CEC Model 21–201) has been described by Brown, Nier, and van Norman (4) and further modifications of the apparatus have been described by Johnston and Brown (11).

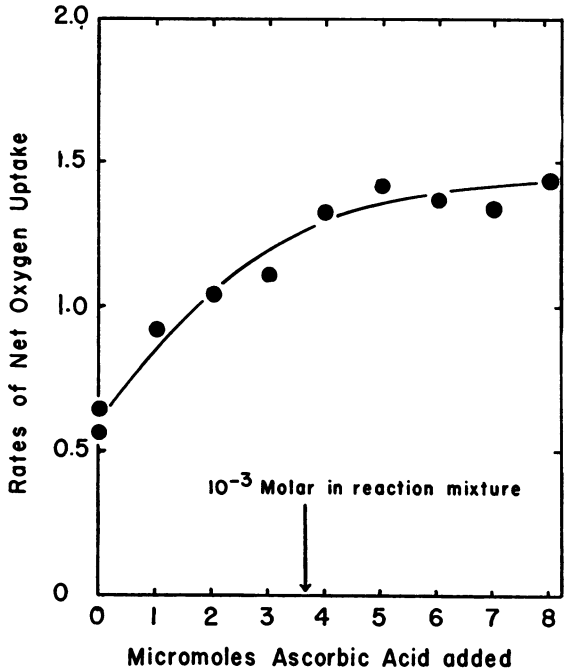
The gas phase for all manometric experiments was air. In experiments using tracer oxygen, the vessel containing 3 cc chloroplast suspensions plus other components of the reaction mixture was attached to the leak assembly of the mass spectrometer and flushed with helium for 5 minutes. Oxygen enriched with mass 34 ($O^{16}O^{18}$) was then added so that the resulting gas phase was composed of 2 to 3 % oxygen in helium. Rates of oxygen consumption and production were calculated from concentrations of mass 32 (ordinary oxygen) and mass 34 which were recorded automatically. Methods of calculation and considerations of possible corrections were discussed in a recent paper by Brown and Weis (5).

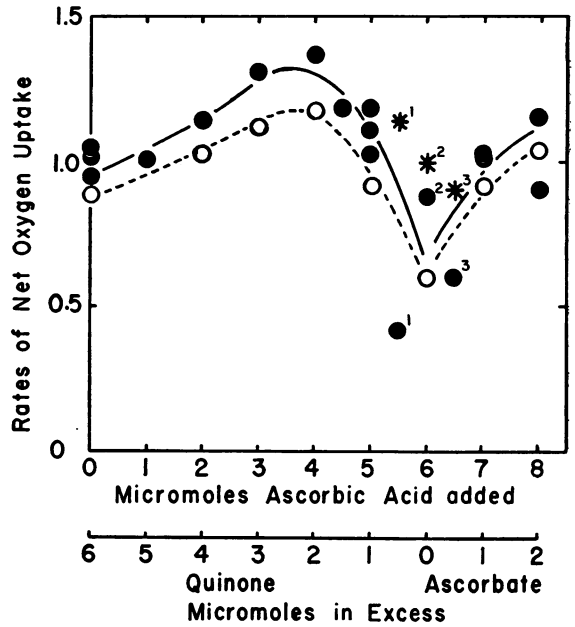
Results

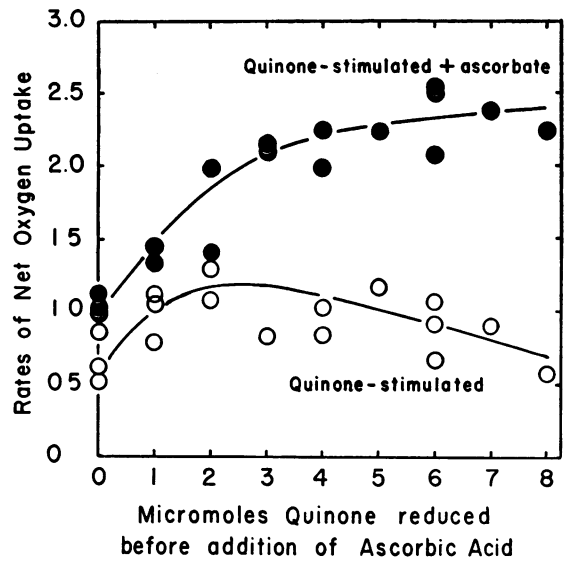
Photooxidation of Ascorbic Acid Coupled With the Mehler Reaction: I. *Effect of ascorbic acid concentration on rates of net oxygen uptake:* The addition of ascorbic acid to chloroplast reaction mixtures containing catalase and ethanol resulted in accelerated rates of net oxygen uptake in the light. When an excess of oxygen equivalent to the added ascorbic acid had been consumed, rates of oxygen uptake reverted to control values. The rates of oxygen uptake during photooxidation of ascorbic acid increased with the amount of ascorbic acid present at the beginning of illumination up to 4 μmoles per vessel (ca 10^{-3} M) (fig 1). With the chloroplast density used, further increases in ascorbic acid concentration resulted in no further increase in rates.

II. *Effects of quinone on rates of photooxidation of ascorbic acid by Mehler reaction mixtures:* Three variations of experimental procedure were used to test for effects of added ascorbic acid on rates of the Mehler reaction in the presence of quinone. In the first, quinone and ascorbic acid were mixed in the dark before adding chloroplasts, catalase, and ethanol. The amount of quinone added was held constant (6 μmoles) while the amount of ascorbic acid added to a series of vessels was varied (0–8 μmoles). The reaction between the ascorbic acid and quinone was completed before addition of the other components of the reaction mixture. It might be expected that only the stimulant present in excess would have an accelerating effect on the subsequent chloroplast reaction in the light. In each case it was possible to compare the rate of net oxygen uptake with rates observed in other experiments (in which aliquots of the same chloroplast preparation had been used) where only the equivalent amount of quinone *or* ascorbic acid was present in the reaction mixture. Both the observed rates for each mixture of quinone and ascorbic acid and the predicted rates are plotted in figure 2. In general, observed rates with the mixtures were slightly higher than those expected from experiments in which quinone or ascorbic acid alone was added to the reaction mixture. The greatest discrepancies were observed when equivalent or nearly equivalent amounts of quinone and ascorbic acid had been mixed. In these cases, two distinct rates were observed: the first, (a low rate) agreed with the rate expected from a reaction mixture containing neither quinone nor ascorbic acid; about 30 minutes after the beginning of illumination a second, higher rate attributable to ascorbate was observed (as much as twice the initial rate) which continued until oxygen uptake ceased at the end of the period of illumination. The discrepancy between observed and expected rates of net oxygen uptake may in part be explained by the action of hydroquinone. Mehler (19) reported a small stimulation of net oxygen uptake by this substance. Although a small stimulation of the Mehler reaction by hydroquinone was found in these experiments, its presence had no effect on the rates of oxygen uptake in reaction mixtures containing ascorbic acid (see below).

In a second variation of these experiments, ascorbic acid was added to the reaction mixture after the completion of quinone reduction. Quinone (in amounts varying from 0–8 μmoles) was equilibrated with chloroplasts, catalase, and ethanol in the dark. A fixed amount of ascorbic acid (6 μmoles) was tipped from the sidearm of each vessel after a period of illumination in which quinone reduction was completed and a period of quinone-stimulated Mehler reaction was followed. Rates of the quinone-stimulated Mehler reaction increased with increasing concentrations of quinone and were maximally stimulated in reaction mixtures in which 2 μmoles of quinone had been reduced in the light. These stimulated rates were approximately twice control rates. At higher quinone concentrations the rates of net oxygen uptake following quinone reduction showed marked inhibition







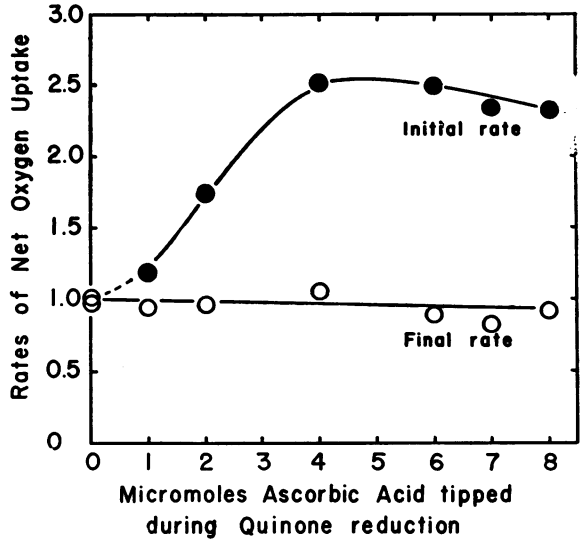


FIG. 1. (*upper left*). Relationship between amounts of ascorbic acid initially present in Mehler reaction mixtures and observed rates of net oxygen uptake (μl O_2/min/vessel). Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 0 to 8 μmoles ascorbic acid.

FIG. 2. (*upper right*). Rates of net oxygen uptake by Mehler reaction mixtures in which ascorbic acid and quinone were mixed before addition of chloroplasts. Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles quinone, 0 to 8 μmoles ascorbic acid. — ● — ● — Observed rates of net oxygen uptake in the light; — ○ — ○ — expected rates; $●^1$. $●^2$, $●^3$ initial rates; $*^1$, $*^2$, $*^3$, final rates (μl/O_2/min/vessel). The initial upward swing represents a relief of the inhibition due to excess quinone; the first peak is due to the maximal quinone effect; and the second upward curve is due to the ascorbate effect. The dip in the middle represents the addition of dehydroascorbate plus hydroquinone which would not be expected to produce stimulation.

FIG. 3. (*lower left*). Rates of net oxygen uptake before and after addition of ascorbic acid to quinone-stimulated Mehler reaction mixtures (μl O_2/min/vessel). Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 0 to 8 μmoles quinone, 6 μmoles ascorbic acid (in sidearm).

FIG. 4 (*lower right*). Initial and final rates of net oxygen uptake in Mehler reaction mixtures following addition of ascorbic acid during quinone reduction (μl O_2/min/vessel). Vessel contents: 3 cc chloroplasts (0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 8 μmoles quinone, 0 to 8 μmoles ascorbic acid.

and stimulated rates at the highest quinone concentrations were only slightly higher than the control rates. In all cases, the rates of net oxygen consumption increased after addition of ascorbic acid from the sidearms of the vessels and these rates increased with the amount of quinone which had previously been reduced by the chloroplasts in the light. Rates of net oxygen uptake after adding ascorbic acid were approximately equal to the rates of oxygen production during quinone reduction at concentrations of quinone above 3 μmoles per vessel. There were no further increases in rates of oxygen uptake in vessels which had initial concentrations of quinone higher than 3 μmoles per vessel (see fig 3). As a control experiment, from 0 to 6 μmoles of hydroquinone were substituted for quinone while the experimental procedure was otherwise the same as that described above. There was a slight stimulation of the Mehler reaction in the presence of hydroquinone, but pretreatment with hydroquinone did not enhance the rates of net oxygen uptake after addition of ascorbic acid to the reaction mixture. After the ascorbic acid tip, rates of net oxygen uptake were approximately twice unstimulated Mehler rates (as would be expected from ascorbic acid acting alone in the system).

In a third variation of the pattern of adding ascorbic acid and quinone, ascorbic acid was added during illumination, but before the completion of quinone reduction. Eight μmoles of quinone were equilibrated with chloroplasts, catalase, and ethanol in the dark. Oxygen production was measured for 25 minutes after the beginning of illumination and then from 0 to 8 μmoles of ascorbic acid in solution was tipped into each reaction mixture from the vessel sidearm. Production of oxygen stopped immediately and oxygen uptake began at rates which exceeded the preceding rates of oxygen production. The duration of these rapid rates of oxygen uptake was, of course, determined by the amount of ascorbic acid added and the amount of unreduced quinone remaining in the reaction mixture at the time of the tip. The final rate of oxygen uptake in each vessel was equal to that of the quinone stimulated Mehler reaction to which no ascorbic acid had been added (see fig 4).

PHOTOOXIDATION OF ASCORBIC ACID COUPLED WITH EXCHANGE REACTION: The Mehler reaction and the exchange reaction are basically the same: they differ only in the manner in which H_2O_2 formed by reduction of molecular oxygen is removed from the reaction mixture. In contrast to the Mehler reaction [in which H_2O_2 is removed through the coupled oxidation of ethanol to acetaldehyde (12)], in the exchange reaction H_2O_2 is dismuted to $H_2O + \frac{1}{2} O_2$ by the endogenous catalase in the chloroplasts (Brown & Good, 3). There is, therefore, no net oxygen uptake because of the exact balance between the oxygen consumed in reaction with the reduced product of photolysis and the production of one-half equivalent of oxygen from decomposition of this product by catalase plus one-half equivalent from the oxidized product of photolysis. Thus, it is not possible to measure the rate of an exchange reaction manometrically and this reaction can be followed only by the use of tracer oxygen. It seemed probable that ascorbic acid oxidation coupled to the exchange reaction would create an imbalance between consumption and production of oxygen which could be measured manometrically. This, in fact, was the case: addition of ascorbic acid to a chloroplast suspension with no other addenda resulted in a small, but measurable, uptake of oxygen in the light and (as in the Mehler reaction) this net uptake of oxygen was greatly enhanced when ascorbic acid was added to the reaction mixture following the reduction of quinone. It was possible, then, to vary the patterns of adding ascorbic acid and quinone and to measure the resulting net oxygen consumption manometrically.

TABLE I

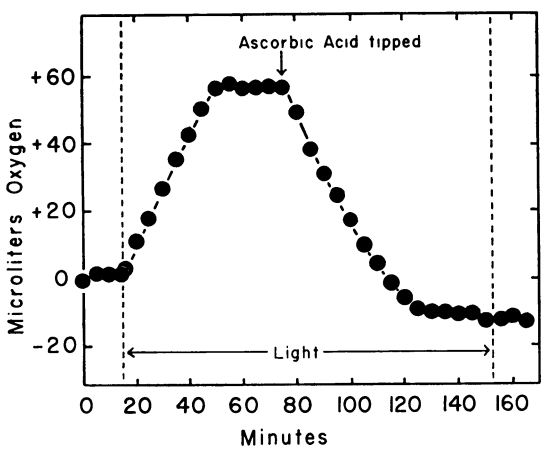
EFFECT ON EXCHANGE REACTION OF QUINONE & ASCORBIC ACID MIXED BEFORE ADDING CHLOROPLASTS

QUINONE/ VESSEL, μmoles	ASCORBIC ACID/VESSEL, μmoles	OXYGEN PRODUCED AFTER ASCORBIC ACID TIP, μl	
		OBSERVED	EXPECTED
6	0	64	67.2
6	2	36	44.8
6	4	25	22.4
6	6	0	0
6	8	0	0

Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 6 μmoles quinone, 0 to 8 μmoles ascorbic acid.

Table I summarizes the results of an experiment in which 6 μmoles of quinone and from 0 to 8 μmoles of ascorbic acid were mixed in the dark before adding the chloroplast suspension. The quantities of oxygen produced on illumination of the reaction mixture agreed closely with the amounts expected (based on the assumption of a quantitative reaction between ascorbic acid and quinone and the absence of any mechanism by which either of these substances could be regenerated). In the vessel containing equivalent amounts of quinone and ascorbic acid, no net uptake or production of oxygen was observed on illumination. In the presence of excess ascorbic acid, there was a slow uptake of oxygen in the light (−0.13 μl/min).

A rapid uptake of oxygen was observed when the addition of ascorbic acid followed the completion of a Hill reaction with quinone (see fig 5). Net oxygen consumption continued only as long as unoxidized ascorbic acid remained in the reaction mixture. The exchange reaction which continued after this point could not be measured manometrically. In a series of such experiments, from 0 to 10 μmoles of quinone were equilibrated with chloroplasts in the dark. After the completion of quinone reduction in the light, 6



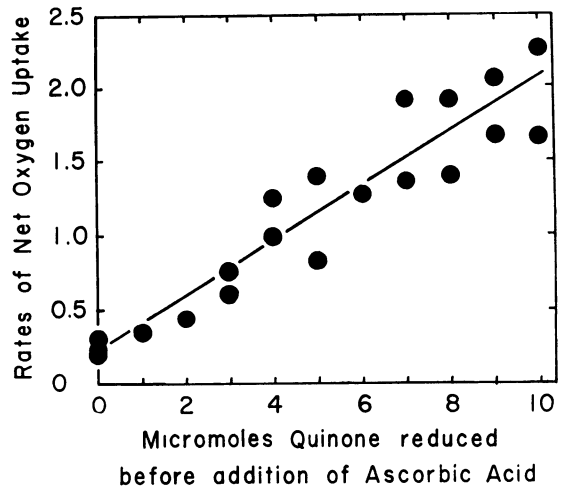


FIG. 5 (*left*). Representative time course of net oxygen uptake: ascorbic acid added to quinone-stimulated exchange reaction. Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll) 6 μmoles quinone, 6 μmoles ascorbic acid (in sidearm).

FIG. 6 (*right*). Dependence of rate of net oxygen uptake during ascorbic acid oxidation on amount of quinone previously reduced by chloroplast preparations. Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 0 to 10 μmoles quinone, 6 μmoles ascorbic acid (in sidearms). Rates expressed as μl O_2/min/vessel.

μmoles of ascorbic acid were tipped from the sidearm of each vessel. As can be seen in figure 6, the rates of net oxygen uptake during photooxidation of ascorbic acid increased linearly over the range of amounts of quinone reduced prior to the addition of ascorbic acid to the system and showed no tendency to saturate.

In a second series of experiments, varied amounts of ascorbic acid (0–8 μmoles) were tipped from the sidearms of the reaction vessels during illumination, after oxygen production equivalent to approximately half reduction of 8 μmoles of quinone had been measured. The contents of the vessel sidearms were tipped without rinsing to add ascorbate as rapidly as possible and to keep protocol comparable to that of mass spectrometer experiments where rinsing was impossible. The expected production or consumption of oxygen after adding ascorbic acid was calculated assuming that 90 % of the contents of the sidearms had been transferred to the main compartment of each vessel. The results of these calculations and the measured volumes of net oxygen production or consumption are summarized in table II. Again there was close agreement between expected and observed values.

TABLE II

EFFECT ON EXCHANGE REACTION OF ASCORBIC ACID TIPPED DURING QUINONE REDUCTION

ASCORBIC ACID IN SIDEARM, μmoles	O_2 PRODUCED BEFORE ASCORBIC ACID TIP μl	TOTAL OXYGEN UPTAKE OR PRODUCTION AFTER ASCORBIC ACID TIP, μl	
		OBSERVED	EXPECTED
0	50	+40	+39.6
2	40	+21.5	+29.4
4	38	+ 8	+11.3
6	38	−20	− 9.1
8	38	−29	−29.0

Vessel contents: 3 cc chloroplast suspension (0.5 mg chlorophyll), 8 μmoles quinone, 0 to 8 μmoles ascorbic acid (in sidearms).

The rates of net oxygen uptake in the light due to unbalanced uptake and production in the exchange reaction were not dependent on the concentrations of ascorbic acid between 2 and 8 μmoles per vessel. In vessels containing ascorbic acid plus 3 cc chloroplast suspension, there were no detectable pressure changes in the dark, but on illumination there was measurable oxygen uptake: with 2 μmoles of ascorbic acid in the vessel the rate of oxygen uptake was 0.16 μl per minute, while with 8 μmoles of ascorbic acid in the vessel the rate of uptake was 0.13 μl per minute. There was no measurable pressure change in these vessels during a dark period following illumination. In vessels containing no ascorbic acid there were no net changes in pressure in the light or in the dark.

It had been assumed that the capacity for light-dependent oxidation of ascorbic acid is associated with the Hill activity of the chloroplast preparations which mediated these reactions. To test this assumption, the rates of these reactions with active chloroplasts were compared to rates with heat-inactivated preparations (heated for 2 hr at 75° C). Active chloroplasts were freshly thawed and diluted aliquots of the chloroplast preparations used for heat-inactivated controls. Even this relatively long heating did not completely destroy the capacity of these chloroplast preparations to carry out reactions of the Hill type, or to mediate the photooxidation of ascorbic acid (see table III). However, heat treatment did reduce all the activities

TABLE III

EFFECTS OF HEAT-INACTIVATION ON CHLOROPLAST ACTIVITY

CHLOROPLAST SYSTEM	QUINONE RATE, μl/min	% OF ACTIVE RATE	MEHLER OR EXCHANGE RATE, μl/min	% OF ACTIVE RATE	RATE AFTER ASCORBIC ACID TIP, μl/min	% OF ACTIVE RATE
			A. Quinone-Mehler + ascorbic acid			
Active	+1.48	100 %	−0.91	100 %	−3.32	100 %
	+1.46		−0.93		−3.23	
Heated	+0.12	0 %	...*	...*	−0.44	15 %
	−0.12				−0.56	
			B. Mehler + ascorbic acid			
Active	...	...	−1.53	100 %	−2.26	100 %
	...	...	−1.23		−1.94	
Heated	...	...	−0.09	0 %	−0.27	9 %
	...	...	0		−0.11	
			C. Quinone-stimulated exchange + ascorbic acid			
Active	+2.02	100 %	−0.41	100 %	−1.81	100 %
Heated	0.39	19 %	...*	...*	−0.20	11 %

* Unreduced quinone still in vessel.

Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 μmoles quinone (present in A & C), 6 μmoles ascorbic acid, 6 mg catalase (present in A & B), 0.1 cc 50 % ethanol (present in A & B).

measured to a small fraction of those of active chloroplasts, and these experiments support the hypothesis that the capacity of chloroplast preparations to mediate the photooxidation of ascorbic acid (whether or not quinone is added to the reaction mixture) is dependent on their Hill activity.

TRACER EXPERIMENTS TO DETERMINE NATURE OF ASCORBIC ACID EFFECTS: The manometric experiments described above demonstrated that light-dependent oxidation of ascorbic acid by chloroplast preparations resulted in accelerated oxygen uptake. It was necessary to utilize tracer techniques, however, to determine whether stimulated rates of net oxygen consumption after addition of ascorbic acid are independent of the products of photolysis, i.e. superimposed on the oxygen uptake and production associated with Mehler and exchange reactions; or whether ascorbic acid is oxidized by the oxidized product of photolysis (or another precursor of oxygen).

Aliquots of the same chloroplast preparation were used in all the experiments described below. Each reaction mixture contained 0.21 mg chlorophyll and experimental conditions such as concentration and volume of other components of the reaction mixtures, intensity of illumination, temperature, and oxygen tension were held as constant as possible. At least six replicate experiments were run of each of the following types: I. Addition of ascorbic acid to an unstimulated Mehler reaction; II. Addition of ascorbic acid to a quinone-stimulated Mehler reaction, and III. Addition of ascorbic acid to a quinone-stimulated exchange reaction. In all cases, ascorbic acid was added during the period of illumination and after stable rates of light-dependent oxygen consumption and production had been measured for at least 20 minutes.

I. ADDITION OF ASCORBIC ACID DURING UNSTIMULATED MEHLER REACTION: The time course of one representative experiment of this type [showing partial pressure changes of ordinary oxygen (mass 32) and tracer oxygen (mass 34)] is illustrated by figure 7. The average rates calculated from six such experiments are summarized in table IV. These ex-

TABLE IV

EFFECTS OF ADDING ASCORBIC ACID TO UNSTIMULATED MEHLER SYSTEM

	UPTAKE, μl/min	PRODUCTION, μl/min	NET, μl/min
Unstimulated Mehler rates	−1.33 ± 0.33*	+0.64 ± 0.14	−0.75 ± 0.24
Rates after adding ascorbic acid	−1.96 ± 0.18	+0.72 ± 0.14	−1.25 ± 0.18
% of rate before adding ascorbic acid	148 %	113 %	167 %

* Mean of six determinations ± standard deviation of mean.

Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles ascorbic acid (in sidearms).

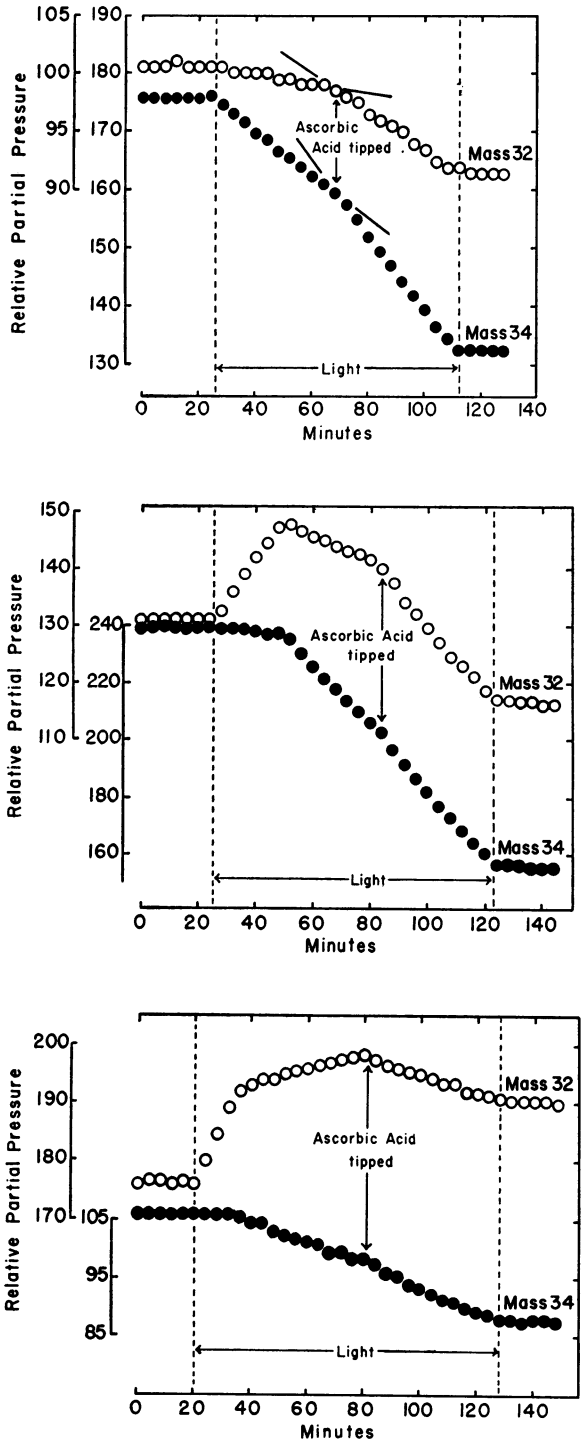


FIG. 7. Effect of addition of ascorbic acid on time courses of normal and tracer oxygen in the Mehler reaction. Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles ascorbic acid (in sidearm). To convert partial pressure units of the ordinate to microliters oxygen multiply by 3.02 for mass 32, by 1.00 for mass 34.

FIG. 8. Time courses of normal and tracer oxygen during quinone reduction, quinone-stimulated Mehler reaction and photooxidation of ascorbic acid. Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles quinone, 6 μmoles ascorbic acid (in sidearm). To convert partial pressure units of the ordinate to microliters oxygen multiply by 3.27.

periments showed that increases in rates of net uptake of oxygen after adding ascorbic acid to Mehler reaction mixtures resulted from two changes in the pattern of oxygen metabolism: rates of both uptake and production of oxygen were accelerated. After adding ascorbic acid to the reaction mixture, rates of oxygen production increased by 13 % while rates of oxygen consumption increased by 48 %. Because rates of both uptake and production of oxygen were changed (rather than only the rate of oxygen consumption) it is more probable that ascorbic acid is oxidized via the intermediary reactions of the products of photolysis, rather than via chloroplast-mediated oxidation which is independent of the Hill activity of the chloroplasts.

II. ADDITION OF ASCORBIC ACID DURING QUINONE-STIMULATED MEHLER REACTION: In this group of experiments quinone was equilibrated in the dark with the components of the Mehler reaction mixture to insure maximum quinone stimulation (8). The time course of a representative experiment of this group is shown in figure 8. At the beginning of illumination, there was an increase in the relative partial pressure of mass 32, while there was no change in partial pressure of mass 34. This indicated that only quinone was being reduced by the reduced product of photolysis. As soon as the reduction of quinone was complete, net uptake of both mass 32 and mass 34 began. Adding ascorbic acid to the reaction mixture resulted in a marked increase in the rate of uptake of mass 32. By comparison, the change in slope of the mass 34 curve was very small. After addition of ascorbic acid, rates of net oxygen uptake increased an average of 91 %. This change in net rate of oxygen uptake was the consequence of a 32 % increase in oxygen uptake and a 46 % decrease in the rate of oxygen production (see table V). Once again, the more plausible mechanism of ascorbic acid oxidation was its reaction with the oxidized product of photolysis or some intermediate in the pathway to oxygen production.

III. ADDITION OF ASCORBIC ACID DURING QUINONE-STIMULATED EXCHANGE REACTION: As in the previous set of experiments, quinone was equilibrated with chloroplasts in the dark. At the beginning of

FIG. 9. Time courses of normal and tracer oxygen during quinone reduction, quinone-stimulated exchange, and photooxidation of ascorbic acid. Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 μmoles quinone, 6 μmoles ascorbic acid (in sidearm). To convert partial pressure units of the ordinate to microliters oxygen multiply by 2.86.

TABLE V

EFFECTS OF ADDING ASCORBIC ACID TO QUINONE-STIMULATED MEHLER SYSTEM

	UPTAKE, μl/min	PRODUCTION, μl/min	NET, μl/min
Quinone-stimulated Mehler rates	−3.19 ± 0.19*	+1.42 ± 0.21	−1.79 ± 0.08
Rates after adding ascorbic acid	−4.20 ± 0.28	+0.77 ± 0.15	−3.42 ± 0.21
% of rate before adding ascorbic acid	132 %	54 %	191 %

* Mean of six determinations ± standard deviation of mean.
Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μmoles quinone (equilibrated in dark with reaction mixture), 6 μmoles ascorbic acid (in sidearms).

illumination, partial pressure changes characteristic of quinone reduction were observed (for time course of a typical experiment see fig 9). On completion of quinone reduction, the balanced oxygen uptake and production of the exchange reaction began. In this reaction the rate of oxygen uptake (due to reduction of oxygen by the reduced product of photolysis) is just balanced by the production of one-half equivalent of oxygen by the action of endogenous catalase on the peroxide thus formed and a second one-half equivalent from the oxidized product of photolysis. Both mass 32 and mass 34 are consumed in the initial reaction with the reduced product of photolysis. Oxygen produced by the action of endogenous catalase is composed of mass 32 and mass 34 in the proportions of their occurrence in the oxygen consumed; the oxygen produced from the oxidized product of the splitting of water is almost entirely mass 32 because of the overwhelming preponderance of H_2O^{16} over H_2O^{18} in the milieu. Hence, during the exchange reaction (in which there is no net change in the partial pressure of oxygen) there is an increase in the relative partial pressure of mass 32 and a decrease in the relative partial pressure of mass 34. On addition of ascorbic acid there was a change from net production to net uptake of mass 32 accompanied by a slight increase in rate of net uptake of mass 34. The average rates for six replicate experiments are summarized in table VI. These data provide the most convincing proof of the hypothesis that ascorbic acid is oxidized via the oxidized product of the splitting of water: the rate of oxygen production decreased by 47 % on addition of ascorbic acid to the reaction mixture. This decrease in oxygen production from the oxidized product of the splitting of water is apparent from the marked change in slope of mass 32 (from production to consumption). Uptake of mass 34 was only slightly accelerated following the ascorbic acid tip.

DISCUSSION

Viewed as a whole, these experiments show that accelerated net oxygen uptake by illuminated chloroplasts on addition of ascorbic acid is the result of two simultaneous changes, i.e. the change in net rate is the result of changes in both uptake and production of oxygen. The unequal effects of ascorbic acid on rates of uptake and production can be explained by a single reaction mechanism: the oxidation of ascorbic acid by an intermediate on the oxidized side of photolysis. This diversion of a portion of one of the products of the splitting of water does not completely block the production of oxygen, and thus there is evidence of a competition between ascorbic acid and the normal enzymatic pathway to oxygen. The proposed mechanism explains both the increased rates of oxygen uptake observed in all cases and the accelerated rates of oxygen production on addition of ascorbic acid to an unstimulated Mehler reaction. If the products of photolysis are produced more rapidly than they can be transported along their normal enzymatic pathways, then either of the following conditions would result: A. A back reaction (or recombination of the oxidized and reduced products of the splitting of water) could remove an appreciable fraction of these products from the system; or B. If only one part of the enzyme system were limited, one of the products of photolysis or a subsequent intermediate might accumulate. Oxidation of ascorbic acid by the oxidized product of the splitting of water could affect either of the above conditions: more efficient removal of this product would decrease the

TABLE VI

EFFECT OF ADDED ASCORBIC ACID ON QUINONE-STIMULATED EXCHANGE

	UPTAKE, μl/min	PRODUCTION, μl/min	NET RATE, μl/min
Quinone reduction	−0.12 ± 0.18*	+2.97 ± 0.27	+2.86 ± 0.33
Quinone-stimulated exchange	−2.69 ± 0.39	+2.48 ± 0.26	−0.11 ± 0.08
After adding ascorbic acid	−3.62 ± 0.39	+1.32 ± 0.16	−1.15 ± 0.16
% of rate before adding ascorbic acid	135 %	53 %	...

* Mean of six determinations ± standard deviation of mean.
Vessel contents: 3 cc chloroplast suspension (0.21 mg chlorophyll), 6 μmoles quinone (equilibrated in dark with reaction mixtures), 6 μmoles ascorbic acid (in sidearms).

probability of back reaction by removing from the reaction system any accumulated intermediates in the pathway to oxygen. Acceleration of the oxygen-consuming reaction in all three of the systems studied (unstimulated Mehler reaction, quinone-stimulated Mehler reaction, & quinone-stimulated exchange reaction) indicates that the extent of back reaction of the products of photolysis is reduced. The rate of oxygen production may be calculated from an observed rate of oxygen consumption according to the usual stoichiometry (20). In all three experimental conditions the rate of oxygen production relative to the observed O_2 uptake is decreased by ascorbate. Even in the unstimulated Mehler reaction (where rates of oxygen production actually increased on addition of ascorbic acid), the stoichiometry of the reaction was changed, indicating that some oxygen precursor was being diverted during the oxidation of ascorbic acid.

It has not been established if the light-dependent oxidation of ascorbic acid by chloroplasts is an enzymatic reaction. The absence of a normal type of ascorbic acid oxidase is certainly apparent. Both manometric and tracer oxygen measurements indicated that oxidation of ascorbic acid was in all cases a light-dependent reaction: it occurred only when chloroplast preparations were illuminated and stopped immediately when lights were turned off. It is possible that chloroplasts contain an ascorbic acid photooxidase but it is not necessary to postulate its existence in order to explain the experimental results.

The stimulatory effects of quinone were discussed in a previous paper (8). In the light of recent isolation of naturally-occurring quinones from chloroplasts by Bishop (2) and Crane (6) and the demonstration that such *p*-quinone derivatives are necessary for Hill activity, it is not surprising that quinone should enhance the overall activity of chloroplast reaction systems. Tracer experiments have demonstrated that quinone stimulation does not change the stoichiometry of the Mehler or exchange reactions (8): in both reactions the ratios of uptake to production of oxygen remained the same as those observed in unstimulated reactions. It is possible that the naturally-occurring quinones (or benzoquinone added to the reaction mixture) are directly responsible for ascorbic acid oxidation. Light and Hill activity of the chloroplasts would be required by this reaction mechanism for oxidation of ascorbic acid via a cyclic reoxidation of reduced quinone by the oxidized product of photolysis.

Summary

The light-dependent oxidation of ascorbic acid by chloroplast preparations was investigated using manometric and tracer techniques. Such photooxidations of ascorbic acid by Mehler or exchange reaction mixtures result in increased rates of net oxygen uptake. Both the Mehler and exchange reactions are accelerated by previous reduction of quinone, and their activity in the mediation of ascorbic acid oxidation is similarly enhanced following quinone reduction. Experiments with tracer oxygen indicate that increased net oxygen consumption after adding ascorbic acid to chloroplast reaction systems is the consequence of simultaneous increase in oxygen consumption and decrease in oxygen production. According to the postulated reaction mechanism, oxidation of ascorbic acid results from a reaction with the oxidized product of photolysis via a cyclic oxidation and reduction of either endogenous quinones or benzoquinone added to the reaction mixture.

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VARIATIONS IN STARCH & TOTAL POLYSACCHARIDE CONTENT OF PINUS PONDEROSA NEEDLES WITH FLUORIDE FUMIGATION[1]

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INTRODUCTION

Stoklasa (10) reported that leaves of plants showing symptoms of chronic sulfur dioxide injury have a lower starch content than normal leaves. Haselhoff et al (4) suggested that lowered starch content was associated only with chronic sulfur dioxide injury, since, in the instance of acute injury, the starch level is fixed at the level which existed at the time of the cell destruction. This hypothesis was not experimentally substantiated. Swain (11) stated that repeated mild, daily, non-marking sulfur dioxide fumigations resulted in no reduction in carbohydrate content of the leaves. Katz and Pasternack (6) studied the effect of concentrations of sulfur dioxide insufficient to produce leaf markings and found an increase in sulfur content, but no significant change in starch concentration.

No similar studies have been found in the literature relating (a) starch and polysaccharide levels in plant leaves with (b) fluoride fumigation. This work was undertaken to provide information on the relation between (a) starch and polysaccharide content of Pinus Ponderosa needles and (b) fluoride exposure level and sequence of fumigation.

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EXPERIMENTAL

FLUORIDE FUMIGATION PROCEDURES: Five groups of ponderosa pine (*Pinus ponderosa* Laws) were