LIGHT-DEPENDENT OXYGEN METABOLISM OF CHLOROPLAST PREPARATIONS. I. STIMULATION FOLLOWING QUINONE REDUCTION[1,2]

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The term "Hill reaction" has been used to describe any light-dependent chloroplast reaction in which a suitable oxidant reacts with the reduced product of the splitting of water and in which oxygen is evolved from the oxidized product. Mehler (7) first showed that oxygen could function as a Hill reagent, i.e., as an oxidant which is effective in promoting photochemical oxygen production by chloroplast preparations. In Mehler's experiments, chloroplast preparations to which excess catalase and ethanol had been added as a sink for H_2O_2 (6) reduced molecular oxygen to hydrogen peroxide in the light while they simultaneously evolved oxygen derived from water. The peroxide was removed by a coupled oxidation of ethanol to acetaldehyde. In this reaction, then, there was for each mole of oxygen reduced an accompanying production of 0.5 mole yielding a net uptake of 0.5 mole. This stoichiometry was confirmed by the tracer experiments of Mehler and Brown (9).

If catalase and ethanol were omitted and the chloroplasts illuminated without addenda, no net oxygen consumption was observed. However, using labeled oxygen in this system Brown and Good (1) showed that the chloroplasts consumed oxygen from the milieu while at the same time they produced an equivalent amount of molecular oxygen. Both processes were shown to be light dependent. In effect, chloroplasts are able to promote an "oxygen exchange" between the oxygen dissolved in the suspending medium and the oxygen combined as water. The one for one stoichiometry of this exchange reaction could be explained most simply by assuming that the endogenous catalase present in the chloroplasts is sufficient to decompose the peroxide (formed as a product of oxygen reduction) to water and 0.5 molecule of oxygen. This explanation may or may not be correct because reduction of oxygen to water rather than to H_2O_2 has not been ruled out. According to the former scheme, which will be assumed to be correct, in the exchange reaction each mole of oxygen reduced by chloroplast preparations in the light is exactly balanced by the production of 0.5 mole from the (OH)-product of photolysis and 0.5 mole from the decomposition of peroxide.

Mehler (8) found that in the presence of catalase and ethanol the rates of net oxygen uptake by chloroplasts in the light were considerably enhanced when these preparations had previously reduced quinone (Hill reaction). These enhanced rates of net oxygen uptake could be increased still further upon addition of ascorbic acid to the reaction system. These observations were confirmed by Good and Hill (3) and also by Habermann and Brown (4).

In the present paper it will be shown that following quinone reduction by chloroplast preparations the rate of the exchange reaction is enhanced in a manner identical in nature to the stimulated Mehler reaction. The enhanced rate is the result of an overall stimulation with no change in reaction stoichiometry. The isotopic tracer method using oxygen labeled with its mass 18 isotope and the mass spectrometer were used to determine the nature of the stimulatory effects of quinone on these reactions.

MATERIALS AND METHODS

Chloroplast preparations were made from the mature leaves of either greenhouse- or field-grown *Phytolacca americana* L. (pokeweed). Leaves were homogenized for three minutes in 0.5 M sucrose in a Waring blendor. The homogenate was strained through several thicknesses of cheesecloth and centrifuged at $1000 \times G$ for 10 minutes in a Servall refrigerated centrifuge. The supernatant was then centrifuged for 20 minutes at $20{,}000 \times G$. Temperature of the rotor during both centrifugations was 0° C. The centrifugate containing intact and fragmented chloroplasts was resuspended in a medium made up of M/15 phosphate buffer (pH 6.8) 0.5 M in sucrose and 0.05 M in KCl. As far as possible all operations were carried out in the dark at temperatures close to 0° C. The prepared chloroplasts were kept frozen in a deep freeze at − 33° C until needed. For use, the suspensions were diluted with the above medium so that they contained the desired concentration of chlorophyll.

Conventional rectangular Warburg vessels were used for both manometric and mass spectrometric experiments. In the former the temperature was 18.8° C, in the latter 23° C. Adaptation of the mass spectrometer (Consolidated Engineering Company Model 21-201) for measurement of metabolic gas exchange has been described by Brown, Nier and Van Norman (2) and further modifications of the apparatus have been described by Johnston and Brown (5). Reaction vessels were illuminated either by a bank of photoflood bulbs placed below or by white fluorescent tubes mounted behind the bath. In the latter case an inclined mirror was so placed that light was reflected onto the bottoms of the vessels. Incident light intensities up to approximately 500 ft-c could be obtained from either source.

The gas phase in all manometric experiments was air. No absorbant for CO_2 was used. CO_2 tension

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was measured during many comparable experiments using the mass spectrometer and no evidence was found for pressure changes caused by CO_2 in any of the chloroplast reactions studied. In experiments with the mass spectrometer the gas phase was a mixture of oxygen of mass 32 and mass 34 in helium. The vessel containing 3 cc of buffered chloroplast suspension plus addenda was first flushed with helium, then oxygen enriched with mass 34 ($O^{16}O^{18}$) was added to the desired concentration of total oxygen (usually 1.5 to 3 %).

Quinone was purified by sublimation of the commercially available chemical. Crystalline catalase (General Biochemicals, Inc.) was used without further purification.

Results

Influence of Pretreatment with Quinone: The rate of oxygen uptake following the reduction of a given amount of quinone was found to depend on whether quinone was added to the reaction mixture before or after the beginning of illumination. In view of this dependence of the extent of stimulation on procedure, standard protocol had to be followed. The reaction mixtures containing quinone were equilibrated in the dark for 10 to 20 minutes before the beginning of illumination to insure maximum stimulation. A standardized procedure did not eliminate variations due to the nature of the different chloroplast preparations. For a given batch of chloroplasts, however, rates were consistent and reproducible.

Dependence of Rates on Initial Quinone Concentration: Figure 1 shows the net oxygen time courses in vessels with varied amounts of quinone added to mixtures of chloroplasts, catalase and ethanol which were otherwise identical in composition. In figure 2 the net rates of oxygen uptake in the Mehler reaction following quinone reduction are plotted against initial quinone concentration. In general, maximally stimulated rates were obtained when the initial quinone concentrations in the reaction mixtures were at least 10^{-3} molar. These observations confirmed with chloroplast preparations from a different source the original observations of Mehler (8).

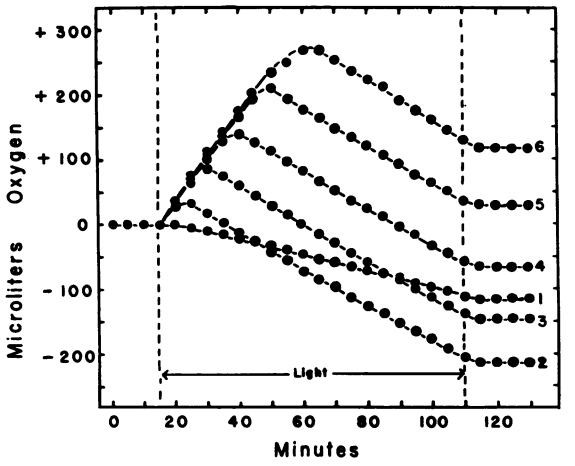

Fig. 1. Time courses of net oxygen production and consumption in vessels containing varied amounts of quinone added to mixtures of chloroplasts, catalase and ethanol of otherwise identical composition. Vessel contents: 3 cc chloroplast suspension (0.4 mg chlorophyll), 4 mg catalase, 0.1 cc 50 % ethanol, quinone as follows: 1. No quinone, 2. 5.5 micromoles, 3. 11.1 micromoles, 4. 16.7 micromoles, 5. 22.2 micromoles, 6. 27.8 micromoles.

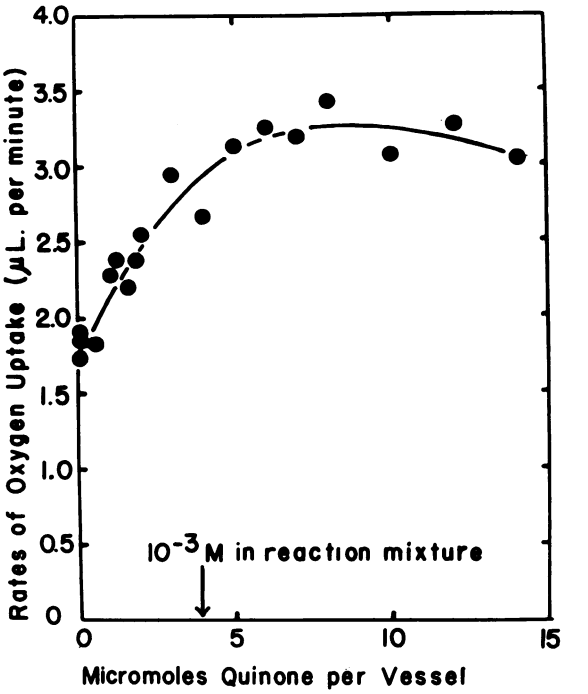

Fig. 2. Effect of initial quinone concentration on rates of net oxygen uptake after completion of quinone reduction. Vessel contents: 3 cc chloroplast suspension (0.4 mg chlorophyll), 4 mg catalase, 0.1 cc 50 % ethanol, 0 to 14 micromoles quinone.

From the known Mehler reaction stoichiometry one might expect that the rates of net oxygen uptake in this reaction would equal the rates of oxygen production with other Hill reagents such as quinone. When maximally stimulated by quinone, rates were usually 2 to 3 times those of the unstimulated Mehler reaction. Even when maximally stimulated, however, observed rates were rarely more than 50 % of those expected from measured Hill rates with identical chloroplast preparations.

Experiments with the Mass Spectrometer: Manometric measurements tell us nothing about the nature of the quinone stimulation illustrated in figure 1, i.e., whether increased net rates are caused by an acceleration of oxygen uptake, an inhibition of production, or changes in the rates of both. The nature of the enhanced rates of net oxygen uptake observed manometrically had not previously been investigated. The effect of quinone reduction on the exchange re-

TABLE I

EFFECTS OF QUINONE REDUCTION ON RATES OF OXYGEN UPTAKE AND PRODUCTION IN THE MEHLER SYSTEM

	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)	NET RATE (μL/MIN)
Unstimulated	1.33 ± 0.33	0.64 ± 0.14	−0.75 ± 0.24
Quinone reaction	0.12 ± 0.18	2.97 ± 0.27	+2.86 ± 0.33
Quinone stimulated	3.19 ± 0.19	1.42 ± 0.21	−1.79 ± 0.08
Percentage of unstimulated rate	239 %	222 %	238 %

Vessel contents: 3 cc chloroplast suspension (containing 0.21 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 micromoles quinone (in those vessels containing quinone). Means and standard deviations of the means represent 6 determinations.

action could not be examined manometrically because in this reaction no net pressure changes are observed. Data obtained with tracer oxygen and the mass spectrometer showed that the exchange reaction and the Mehler reaction are accelerated in the same way following quinone reduction.

a. Effects of previous quinone reduction on the Mehler reaction: Table I summarizes the results of 12 experiments. In each experiment the vessel contained 3 cc chloroplast suspension (0.21 mg chlorophyll), 0.1 cc 50 % ethanol and 6 mg catalase. In half the experiments 6 micromoles quinone were present in the reaction mixture (equilibrated in the dark for 20 minutes before the beginning of illumination); in the other half, no quinone was present in the vessels.

The average rate of oxygen uptake after quinone reduction was 239 % of the unstimulated rate, while the rate of oxygen production was 222 % of the unstimulated rate. Net quinone-stimulated rates were 238 % of the unstimulated rates of net oxygen uptake. Thus, enhanced rates of net oxygen uptake following quinone reduction appeared to result from an overall stimulation, i.e., an enhancement of rates of both uptake and production with no change in the stoichiometry of the Mehler reaction.

b. Effects of previous quinone reduction on the exchange reaction: The exchange reaction was accelerated by previous reduction of quinone by illuminated chloroplasts and, as with the Mehler reaction, the extent of the stimulation was enhanced by a period of dark equilibration of the quinone with the chloroplast preparation (see table II). In experiment 1 quinone was added to the chloroplast preparation after a period of exchange in the light. Chloroplasts and quinone were left in the dark for 10 minutes after the addition prior to the next period of illumination. The rates of oxygen uptake and production (after the quinone had been reduced in the light) were about twice those observed in the light period preceding the addition of quinone. A period of illumination following another dark period showed that the rates did not revert to unstimulated values. In experiment 2 quinone was tipped from the sidearm while the unstimulated exchange reaction was proceeding normally in the light. Quinone reduction, of course, occurred immediately, followed by the stimulated oxygen exchange.

The data in table II are typical of the many experiments which were run. The chloroplast preparations used in these two experiments were not identical and because of the difference in activity of the two preparations comparison of rates is uncertain. Increases in the rates of both uptake and production of oxygen were observed in experiment 2 (112 % and 120 % of unstimulated values). However, the extent of stimulation was only about 0.1 of that observed in experiment 1 where 192 % and 201 % of the unstimulated values were observed following quinone reduction by chloroplasts equilibrated with quinone in the dark.

DISCUSSION

Experiments using the tracer oxygen technique have indicated that the stimulatory effect of quinone reduction on the subsequent net oxygen uptake by illuminated chloroplasts in the presence of excess catalase and ethanol is the result of a simultaneous

TABLE II

EFFECTS OF QUINONE REDUCTION ON RATES OF OXYGEN UPTAKE AND PRODUCTION IN THE EXCHANGE REACTION

	1. QUINONE TIP FOLLOWED BY 10 MINUTES DARK		2. QUINONE TIPPED IN THE LIGHT	
	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)
Unstimulated exchange	2.03	1.85	3.26	2.92
Quinone reaction	0.00	4.15	0.00	5.64
Quinone stimulated exchange	3.90 *	3.72 *	3.65	3.52
Percent of unstimulated rates	192 %	201 %	112 %	120 %

* Average for two light periods.

Vessel contents: 1. 3 cc chloroplast suspension (0.94 mg chlorophyll), 6 micromoles quinone in sidearm. 2.3 cc chloroplast suspension (0.70 mg chlorophyll), 6 micromoles quinone in sidearm.

increase in the rates of both uptake and production of oxygen. An effect, identical in nature, on the related exchange reaction was observed. In both cases quinone reduction which so greatly enhanced reaction rates produced no change in the stoichiometry of the overall reaction.

Although these experiments have further clarified the nature of the quinone stimulation, the mechanism by which this substance acts is still a matter of conjecture. The basis of the net effect observed in the Mehler system is of course the exchange reaction. Thus the quinone effect has to be explained in a way valid for both the exchange system (incorporating the catalatic decomposition of H_2O_2) and the Mehler, or catalase-ethanol-containing system (in which H_2O_2 is removed by the coupled oxidation of ethanol to acetaldehyde). Any explanation also has to take into account the fact that the stoichiometry, i.e., the ratio of oxygen taken up to oxygen produced, does not change.

Since it is a common experience with the photochemical system in plants that whatever happens to the reducing side is reflected in a corresponding change on the oxygen side, we have the following choices of mechanism: (a) The product of quinone reduction may inhibit the back reaction between photochemically produced (H) and (OH) and thus favor the reduction of free oxygen; (b) it may stimulate the evolution of oxygen and thus automatically release more (H) for reduction; and (c) it may catalyze the formation of peroxide from (H) and free oxygen which would as in (b) lead to a proportionately greater rate of oxygen production.

Mehler (8) showed that no stimulation of oxygen uptake resulted when hydroquinone was used in place of quinone. Therefore a simple catalytic reaction in solution based on a shuttle between hydroquinone and quinone on either side of the exchange system is not a sufficient explanation for the observed stimulation.

Quinone must be reduced in the presence of chloroplasts. Because of the enhancement of the quinone effect by equilibration with chloroplasts in the dark prior to the beginning of illumination (and quinone reduction), it seems appropriate to assume that there may be a binding of the quinone at a reactive site on the chlorophyll complex. This still does not allow us to decide, however, whether its effect consists of an inhibition of back reactions or the catalysis of hydrogen transfer.

Summary

Illuminated chloroplasts form hydrogen peroxide in the presence of oxygen by a photoreduction analogous to that of iron salts, quinone and other Hill reagents. Such a reduction of oxygen is accompanied by the photochemical evolution of oxygen from water. Labeling of the molecular oxygen with O^{18} has been the means for distinguishing between uptake and release of this gas and this method has been employed again in the present study.

Previous observations that the reduction of small amounts of quinone by illuminated chloroplasts accelerated net oxygen uptake in the Mehler system have been confirmed. It has been shown that stimulation by quinone does not change the ratio of uptake to production of oxygen. This holds for the Mehler system and for the simpler oxygen exchange system as well.

Possible mechanisms for the quinone effect have been discussed.

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