COMPARISON OF THREE PHOTOCHEMICAL ACTIVITIES OF CHLOROPLASTS 1, 2

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The Hill reaction is one of the simplest chloroplast reactions in which one can measure in unambiguous terms both the reducing and the oxidizing equivalents produced in the process of photosynthesis. In its simplest aspect, the oxidizing power is measured as oxygen production, while the reducing power is measured in terms of reduction of some electron acceptor (9). Two other photochemical reactions catalyzed by plant chloroplasts are the photochemical oxidation of ascorbic acid in the presence of the dye 2,6-dichlorophenolindophenol (DPIP), and the photochemical reduction of the dye indigo carmine (IC), which photoreduction requires the presence of both ascorbic acid and DPIP (10, 12). These reactions also can be described in terms of the classical Hill reaction, in which case one could consider the ascorbate-DPIP couple as reacting with the photochemical oxidizing system, allowing the photochemical reducing system to react with oxygen and indigo carmine respectively (10). Although there is to date no decisive experimental proof to show that such is the case, isotopic evidence does support this hypothesis (4). This paper describes the effect of various chloroplast treatments upon the ability of chloroplasts to catalyze a conventional Hill reaction, the photochemical oxidation of ascorbic acid in the presence of DPIP, and the photochemical reduction of indigo carmine, the purpose being to see if one can in this manner correlate these 3 photochemical activities of the chloroplast.

METHODS AND MATERIALS

The dye, 2,6-dichlorophenolindophenol, was obtained from Eastman Organic Chemical Co., Rochester, New York. Indigo carmine dye was obtained from National Aniline Division, Allied Chemical and Dye Co., New York, New York. Tris(hydroxy-methyl) amino methane was obtained from Sigma Chemical Co., St. Louis, Mo. Digitonin was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. All other chemicals were obtained from commercially available sources and were of reagent grade. Water low in ion content was prepared by filtering distilled water through a column, approximately 4 cm in diameter and 28 cm long, filled with Amberlite MB-3 resin.

To prepare chloroplasts, 50 g of washed sugar beet leaves without petioles were blended for 50 seconds in a Waring blender containing 100 ml of buffer solution (0.01 M KCl, 0.02 M phosphate, pH 7.0). After straining this homogenate through cheesecloth, the resulting fluid was centrifuged and the pellet coming down between 200 × G and 4000 × G (containing chloroplasts and chloroplast fragments) was washed once and resuspended in buffer solution.

The digitonin extract of the chloroplasts was prepared by incubating washed chloroplasts in 1% digitonin solution for 15 minutes. Sonic oscillation of the chloroplasts was accomplished by a 10 minute treatment in a 10 K.C. Raytheon sonic oscillator. Ice water was used to keep the chamber cool. For incubation of the chloroplasts at pH 8.5 or pH 5.5, 0.01 M tris(hydroxymethyl) amino methane, pH 8.5, and 0.01 M acetate buffer, pH 5.5 were used. The treatment with water of low ion content consisted of an incubation for 30 minutes. Following these treatments the various suspensions were centrifuged for 10 minutes at 23,000 × G at 0° C. The supernatant solution from the digitonin-treated preparation was used as the digitonin extract. For the other preparations, the pellet was resuspended in buffer solution and used in the various tests.

Photo reduction of DPIP, used as a measure of the Hill reaction, was followed spectrophotometrically at 590 mμ. The reaction mixture consisted of 10 micromoles (µM) of phosphate buffer, pH 7.0, 0.5 µM of DPIP, chloroplast suspension equal to 0.1 mg of chlorophyll and water in a total volume of 3.0 ml.

The photoreduction of indigo carmine was determined essentially as described by Vernon and Hobbs (10), except that phosphate buffer at a pH of 7.0 was used. The reaction mixture contained 0.2 µM of indigo carmine dye, 1.0 µM of DPIP, 10 µM of ascorbic acid, 100 µM of phosphate buffer, pH 7.0, and from 0.1 to 0.2 mg of chlorophyll from the chloroplast preparations.

Photooxidation of the DPIP-ascorbate couple was determined using a modification of the method of Vernon and Ihnen (11), which consisted of titrating the remaining ascorbic acid with an excess of DPIP and using the spectrophotometer to determine the excess DPIP in the illuminated and dark reactions. Excess ascorbic acid was used in the reaction mixture since Vernon and Ihnen showed that the rate of the reaction was considerably greater when an excess was present. The reaction mixture consisted of 10 µM phosphate buffer, pH 7.0, 5 µM DPIP, 10 µM ascorbic acid, and 0.1 mg of chlorophyll from the chloroplast preparations in a final volume of 3.0 ml.

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Chlorophyll was determined by the method of Arnon (1). All spectrophotometric measurements were made with a Beckman Model DU spectrophotometer. The illumination time was 4 to 8 minutes and a light intensity of 4000 ft-c was employed. For each series of experiments a control of similar composition was used. Typical reaction rates for the control reactions are as follows: 1.5 μM IC photoreduced per mg chlorophyll in a 4 minute period, 1.9 μM DPIP reduced per mg chlorophyll in an 8 minute period for the Hill reaction, and 2.6 μM ascorbic acid photooxidized per mg chlorophyll for an 8 minute period.

RESULTS

Of the various chloroplast treatments examined, 2 were found which resulted in extensive inhibition of the Hill reaction, while leaving the IC photoreduction and the photooxidation of ascorbic acid relatively unaffected. Figure 1 presents the results obtained after heating the chloroplast preparation at 51° C for 10 minutes and also following incubation at pH 8.5 for 30 minutes. The ability of chloroplasts to catalyze the Hill reaction was most sensitive to heating at 51° C, but both treatments showed preferential destruction of activity in the Hill reaction. The activity of the system responsible for the Hill reaction at high pH values agrees with data reported by Punnett (8). The possibility that this treatment extracted a factor from the chloroplasts which was necessary for complete activity was examined by adding to the reaction mixture an aliquot of the buffer in which the chloroplasts were incubated. No significant increase in activity was noted. Consequently, it would appear that the incubation at pH 8.5 inactivates some reaction component necessary for the Hill reaction to proceed, but not necessary for the other 2 photochemical reactions to proceed. The component destroyed by these treatments could be operative in the oxygen evolving system.

Four treatments were found which inhibited the IC photoreduction more severely than the other 2 photochemical activities. Sonic oscillation for 10 minutes was most effective in destroying indigo carmine photoreduction activity, as shown in figure 2. Treatment with sodium azide was less striking concerning inhibition of IC photoreduction. Exposure to low water and to a pH of 5.5 also resulted in approximately 50 % inhibition of IC photoreduction while the other 2 photochemical activities were only slightly affected, as shown in figure 3. The stability of the Hill reaction to a short treatment with water of low ion content was to be expected from data previously reported (2, 13). The ability of the system responsible for IC photoreduction to sonic oscillation was surprising, since this treatment did not appreciably affect the ability of the chloroplasts to catalyze the Hill reaction. Sonic oscillation for longer periods of time served to decrease the activity of the chloroplasts in the Hill reaction also, indicating that disruption of the chloroplast beyond a certain stage would prevent the Hill reaction from proceeding. It was not possible to show that any factor necessary for IC photoreduction was extracted by incubation in water of low ion content or at pH 5.5, so apparently such treatments inactivate some component specifically required for IC photoreduction, and again serves to illustrate that the 3 photochemical systems are not entirely similar.

The effect of ZnSO₄ upon the 3 chloroplast activities is shown by figure 4. This was the only case during this investigation that the photooxidation of ascorbic acid was reduced more than the other 2 photochemical activities. This inactivation may be due to displacement by the zinc ion of some other necessary metal ion in this system, or to the ability of zinc ion to complex some compound necessary for this reaction. The slight inhibition of the Hill reaction agrees with results reported by MacDowell, since he found that zinc sulfate prevents the action of many inhibitors (6) and it would not itself be expected to greatly inhibit this reaction.

Treatment of a chloroplast preparation with digitonin, followed by centrifugation, yielded a digitonin extract which was almost devoid of activity in the Hill reaction and IC photoreduction. The digitonin extract, however, was more than 6 times as active in the photooxidation of ascorbic acid on a chlorophyll basis, than the original chloroplasts as shown in figure 5. This can be compared with the report of Nieman and Vennesland (7), that digitonin extracts of chloroplasts are capable of catalyzing a photooxidation of cytochrome c in the presence of oxygen. In terms of enzymatic complexity it would be expected that both the photooxidation of ascorbic acid and the photooxidation of cytochrome c would be much more simple than the system necessary for both the Hill reaction and IC photoreduction. Apparently the digitonin ruptures the photosynthetic apparatus, leaving only a primitive system capable of functioning in photooxidations.

In addition to the treatments listed above, there were several chloroplast treatments which resulted in about equal destruction of the ability of the chloroplasts to catalyze the Hill reaction and IC photoreduction, while leaving the photooxidation of ascorbic acid almost unaffected. Incubation with 8-hydroxyquinoline at 10⁻³ M final concentration resulted in a decrease of about 20 % in ability to carry out the Hill reaction and photoreduce IC. Digestion with lipase resulted in a 50 % decrease in these activities, while digestion with pancreatin caused a loss of about 70 % for these 2 activities. The treatments mentioned above did not appreciably affect the photooxidation of ascorbic acid. Incubation with phenyl mercuric acetate completely inhibited the activity in the Hill reaction and IC photoreduction, while affecting the photooxidation of ascorbic acid only to the extent of a 40 % inhibition. Digestion with lysozyme, or incubation with 2,4-dinitrophenol caused only a slight inhibition for all 3 activities.
Fig. 1. Treatments preferentially inhibiting the Hill reaction. The solid bar represents data obtained upon heating chloroplasts for 10 minutes at 51°C. The open bar represents data obtained upon incubation of chloroplasts for 30 minutes at pH 8.5 as described in the section on Methods.

Fig. 2. Treatments preferentially inhibiting the photoreduction of indigo carmine. The solid bar represents data obtained after chloroplast exposure to sonic oscillation for 10 minutes. The open bar represents data obtained upon addition of sodium azide at a final concentration of $10^{-3}$ M to the reaction mixture.

Fig. 3. Treatments preferentially inhibiting the photoreduction of indigo carmine. The solid bar represents data obtained following incubation of chloroplasts in water of low ion content for 30 minutes. The open bar represents data obtained upon incubation of chloroplasts at pH 5.5 for 30 minutes as described in section on Methods.

Fig. 4. The effect of zinc ion upon the 3 photochemical activities. ZnSO$_4$ at a final concentration of $10^{-3}$ M was added to the standard reaction mixtures.
DISCUSSION

The intent of the present investigation was to see if the 3 photochemical reactions followed were similarly affected by various treatments of the chloroplasts, in order that the degree of similarity of the 3 reactions could be assessed. From the data presented, it appears that they undoubtedly differ in some of the components involved in the reactions. In general, it appears that the photochemical oxidation of ascorbic acid in the presence of DPIP is the least sensitive of the 3 reactions, being less affected by all the treatments. Only addition of zinc ion resulted in less ability to photooxidize ascorbic acid, when compared to the other photochemical activities. This general insensitivity is further emphasized by the activity obtained in digitonin extracts of chloroplasts. Such preparations lost almost completely the ability to catalyze the Hill reaction and the photoreduction of IC, but their ability to catalyze the photooxidation of ascorbic acid was markedly increased. This would be in accord with the proposed mechanisms for the photochemical oxidation of ascorbic acid (12), in which the oxidative system produced in the primary photochemical reaction is thought to react with reduced DPIP, which in turn becomes reduced again by interaction with the ascorbate. In the proposed mechanism, the reducing power would be simultaneously causing the reduction of oxygen to the level of hydrogen peroxide. Thus, the enzyme systems involved in oxygen evolution would not be involved. Likewise, the interaction of oxygen with the photochemical reducing power would probably be through the mediation of riboflavin-5'-phosphate or some related compound, which would be relatively insensitive to the treatments used here. It would appear that the photooxidation of ascorbic acid in the presence of DPIP requires only the chlorophyll system responsible for the initial photochemical act of photosynthesis and a minimum of associated enzymes.

The photoreduction of IC and the Hill reaction are both much more sensitive to the various treatments described than is the photooxidation of ascorbic acid. However, with the treatments described in this investigation, it is possible to preferentially inhibit either of these 2 reactions. In looking for an explanation of this behavior, one should consider that the salient differences between the Hill reaction and the IC photoreduction reaction are 1) the requirement for the presence of DPIP and ascorbic acid for IC photoreduction and 2) the difference in the oxidation potentials for the electron acceptors used in these reactions. The E° for IC is $-0.125$ volts, while the $E°$ for DPIP is $0.217$ volts at pH 7.0 (5).

The distinction between the Hill reaction and IC photoreduction, as shown by the treatments described in this investigation, could be due to interaction of IC and DPIP at different points in the photochemical reducing system. The potential difference in the 2 compounds would make this both possible and plausible. Another possibility for the differential effect of the chloroplasts treatments would concern the oxygen evolution system. If the DPIP and ascorbate are interacting with the photochemical oxidizing system as postulated (10), then those treatments which preferentially inhibit the Hill reaction could be destroying the enzyme system involved in oxygen evolution. From the experimental data available, it is not possible to say which of these possibilities is operative in each case. From the differential inhibition of the 2 reactions, one can say that there are different components involved in the 2 photochemical systems responsible for the Hill reaction and for IC photoreduction, but it is not possible to localize these different components.

**Summary**

1. The ability of chloroplasts to catalyze 3 photochemical activities following various treatments has been determined. The 3 photochemical activities determined were 1) the Hill reaction with 2,6-dichlorophenolindophenol (DPIP) as electron acceptor 2) the photoreduction of indigo carmine (IC) in the presence of ascorbate and DPIP and 3) the photochemical oxidation of ascorbate in the presence of oxygen and DPIP.

2. Heating chloroplasts at 51° C, and incubation at pH 8.5 for 30 minutes caused a preferential destruction of activity in the Hill reaction.

3. Sonic oscillation for 10 minutes, addition of sodium azide, incubation in ion low water, or incuba-

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**Fig. 5.** Activity of digitonin extract in the 3 photochemical reactions. See section on Methods for details of preparation of digitonin extract.
tion at pH 5.5 resulted in preferential destruction of IC photooxidation ability.

4. Addition of zinc ion preferentially inhibited the photooxidation of ascorbic acid.

5. Treatment of chloroplasts with digitonin resulted in a drastic loss of ability to catalyze the Hill reaction and IC photooxidation, yet yielded a preparation which was over 6 times as active in terms of ascorbic acid photooxidation.

6. These results are briefly discussed in terms of reaction mechanisms for these 3 photochemical reactions.

LITERATURE CITED


UNCOPPLERS OF SPINACH CHLOROPLAST PHOTOSYNTHETIC PHOSPHORYLATION 1, 2, 3

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It was recently observed by Arnon et al (3) that the formation of ATP could occur during the course of the Hill reaction, with either ferricyanide or TPN as the Hill oxidant. The formation of ATP was stoichiometric with and dependent on the transfer of electrons to the Hill oxidant. The converse obtained to some extent also; that is, the Hill reaction with ferricyanide as oxidant proceeded approximately twice as fast if the phosphorylating reagents were present. We were able to repeat these observations of Arnon et al, including both aspects—ATP formation dependent on simultaneous electron flow, and electron flow stimulated by simultaneous phosphorylation (4). By using somewhat different conditions we found the stimulated rate of ferricyanide reduction to be from 3 to 3.5 times that of the control rate (7).

In investigating the mechanism by which this coupling occurs it may be useful to find reagents or physical treatments that lead to a disruption of the mechanism—i.e., uncouplers. A compound that is an uncoupler in this system, we will define as one that permits electron flow to proceed at a rapid rate in the absence of phosphorylation. Specifically it should meet the following criteria: a) in the phosphorylating Hill reaction, the reagent will inhibit the formation of ATP; b) uncouplers would presumably be able to inhibit electron flow in other coupled systems.