Activities of Chloroplast Fragments

I. HILL REACTION AND ASCORBATE-INDOPHENOL PHOTOREDUCTIONS*

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SUMMARY

Hill activity with ferricyanide and nicotinamide adenine dinucleotide phosphate and photoreduction of NADP and methyl red supported by ascorbate and 2,6-dichloroindophenol were studied as a function of the size of chloroplast fragments.

Sonic treatment of chloroplasts resulted in a decrease in Hill activity with NADP and ferricyanide. The decrease was accompanied by a change in the dependency of the reduction rate upon ferricyanide concentration and light intensity. With either electron acceptor, the Hill activity was highest with fragments of medium size and decreased with decreasing size of the fragments.

The photoreduction of NADP and methyl red with ascorbate and 2,6-dichloroindophenol increased after sonic treatment. The larger fragments were less active than the sonically disrupted chloroplasts, whereas the small fragments showed a very high activity; that is, they were several times more active than the larger fragments or sonically treated chloroplasts. Evidence is presented to indicate that the increase in the activity induced by sonic treatment was due to an increased accessibility of the electron-donating reaction.

The first comparison of the Hill activity of chloroplast fragments of various sizes was reported by Thomas et al. (1) who attempted to substantiate the concept of a photosynthetic unit proposed by Emerson and Arnold (2) from a kinetic analysis of photosynthesis. Recently, Park and Pon (3) observed a rather uniform distribution of ferricyanide Hill activity among chloroplast fragments of differing size. Since the activities of the various fragments were comparable to that of whole chloroplasts, they suggested that the “quantasome” was the minimum unit of photosynthesis (3). In contrast, Becker et al. (4, 5) found that the relatively large fragments, sedimented by centrifugation between 20,000 and 50,000 $\times g$, exhibited higher Hill activity than any of the smaller fragments. They assumed, therefore, that the Hill activity was not related simply to fragment size but rather to a chemical component, or the molecular environment, of the fragments (5). More recently, Izawa and Good (6) found that disruption of the chloroplast structure by sonic treatment had two effects on the Hill activity, an uncoupling of the electron transfer system from phosphorylation and loss of the Hill reaction itself. The rate of the Hill reaction, determined in the presence of an uncoupler, methylamine, decreased with decreasing fragment size, and the activity of the smallest fragments was only 5% of that of the untreated chloroplasts (6).

We have shown previously that sonic treatment of chloroplasts resulted in a rapid and complex, pH-dependent change in the Hill activity with ferricyanide and the plastocyanin-restored nicotinamide adenine dinucleotide phosphate photoreduction. Following the rapid phase which occurs during the first few minutes of sonic treatment, the activities remained rather constant and insensitive to ultrasound. The difference in kinetics between the activity changes and fragmentation of the chloroplasts suggested to us that the activity was relatively independent of the size of the chloroplast fragments (7, 8).

In order to test this view and, at the same time, to investigate the apparent contradiction in the literature, the activities of chloroplast fragments obtained by fractional centrifugation were re-examined. It was previously shown that conditions necessary for maximal activity with sonically treated chloroplasts were significantly different from those described for untreated chloroplasts; for example, a difference in the pH required for maximal activity (8). These earlier observations suggested that the Hill and photoreduction activities of the fragments were actually higher than reported by other investigators who assayed the untreated chloroplasts and the fragments under the same conditions. Therefore, a further characterization of the assay system for maximal activity with sonically treated chloroplasts was undertaken.

Thus far, only the Hill reaction with ferricyanide or quinone has been determined with chloroplast fragments, and these reactions might well be catalyzed only by oxygen-evolving systems in sonically treated chloroplasts (8). In the present report, however, an additional activity, the photoreduction of NADP on addition of plastocyanin, was also determined as a function of the size of the chloroplast fragments. With the use of ascorbate and 2,6-dichloroindophenol as an alternate electron donor to water, it was possible to determine the activity associated only with the long wave length pigment system (9), sep-
respectively, is used. The subscript indicates the role played by the compound. Photosystem 2, the short wave length pigment system, or oxygen-evolving system, of chloroplasts; CMU, 3-[(p-chlorophenyl)-1,1-dimethylurea.

The abbreviations used are: DCI, 2,6-dichloroindophenol; Photosystem 1, the long wave length pigment system of chloroplasts; Photosystem 2, the short wave length pigment system, or oxygen-evolving system, of chloroplasts; CMU, 3-[(p-chlorophenyl)-1,1-dimethylurea.

Fig. 1. Effect of ferricyanide concentration of Hill activity with untreated and sonically treated chloroplasts. The untreated and sonically treated chloroplasts (sonicated) were assayed at pH 7.8 and 6.0, respectively. Each reaction mixture contained chloroplasts equivalent to 17.7 μg of chlorophyll, and the time of illumination was 30 sec.

The distribution of the activities among the chloroplast fragments of varying size, as well as the nature of the sonically induced change in the activities, is presented. These results support the previous assumption that the activities and the size of the chloroplast fragments bear no simple relationship to each other. It is also shown that the photoreduction of NADP or methyl red with ascorbate and DCI occurred at a high rate in the smallest chloroplast fragments studied.

Materials and Methods

The preparation of broken chloroplasts from spinach leaves obtained from a local market was described previously (19). Sonic treatment of chloroplasts was carried out in the cold in 0.005 M phosphate, pH 7.8, containing 0.4 M sucrose and 0.01 M NaCl. The sonic treatment time was 10 min, and the sonic oscillator (Raytheon 10-kc) was operated at full power. For the measurement of Hill activity (Fig. 5), methanol was added to the suspension at a final concentration of 15%. Chloroplasts treated with deionized water or sonically treated for 10 min are referred to as untreated and sonically treated chloroplasts, respectively.

Centrifugal fractionation of sonically treated chloroplasts was performed with the Serval model RC-2 refrigerated centrifuge and the Spinco model L ultracentrifuge. The rotors used with the latter centrifuge were the No. 40 for 50,000 × g and the SW 39 for 175,000 × g. All precipitates were suspended in the same medium as that used for sonic disruption. The designation of P and S for precipitate and supernatant solution, respectively, is used. The subscript indicates the centrifugal force, in g × 10⁻⁴, which is used to obtain the fractions.

RESULTS

Oxidant Concentration—In order to compare the activities of sonically treated chloroplasts with untreated chloroplasts, it was necessary to establish assay conditions to measure the maximal Hill and photoreduction activities of sonically treated chloroplasts. It was shown previously that the pH optimum for the Hill activity with ferrocyanide by sonically treated chloroplasts was shifted toward the acid side. It has now been found that sonically treated and untreated chloroplasts exhibit a markedly different dependence on ferrocyanide concentration.

The relationship between Hill activity and ferrocyanide concentration for both sonically treated and untreated chloroplasts is illustrated in Fig. 1. Lumry and Spikes (14) reported that the rate of ferrocyanide Hill activity with untreated chloroplasts was essentially independent of oxidant concentration within the range of 5 × 10⁻⁴ M to 10⁻³ M. This was confirmed in the present study, at least in the range of oxidant concentration from 5 × 10⁻⁴ M to 10⁻³ M. In contrast, the rate of ferrocyanide Hill activity by sonically treated chloroplasts decreased markedly when the concentration of ferrocyanide was decreased below 4 × 10⁻⁴ M. That is, sonically treated chloroplasts exhibited a much lower activity than untreated chloroplasts at low concentrations of ferrocyanide. This finding was rather unexpected since it was felt that disruption of the chloroplast structure would increase the accessibility of the oxidant to the reactive site in the chloroplasts. It may be assumed, therefore, that either ferrocyanide is reduced by sonically treated chloroplasts at a site in the electron transport chain different from that in untreated chloroplasts, or that the component (or site) of the electron transport chain which serves as the reductant for ferrocyanide reduction underwent some modification by sonic treatment.

Light Intensity—A second marked difference in the activity of chloroplasts before and after sonic treatment relates to the effect of light intensity. The relationships between the light intensity and the rate of the Hill reaction with ferrocyanide or NADP photoreduction with ascorbate and DCI as electron donor are shown in Figs. 2 and 3, respectively. In either case, a plot of the reciprocal of the velocity with respect to the reciprocal of the light intensity, as suggested by Lumry and Spikes (14), yielded straight lines with both untreated and sonically treated...
chloroplasts. In this type of plot, the slope and the intercept of the straight line are related, respectively, to the light rate constant and the rate constant for the limiting dark reaction (14).

It is apparent that, for the ferriyanide Hill reaction, both rate constants were affected significantly by sonic disruption of the chloroplasts. On the other hand, sonic treatment resulted in a marked change in the dark rate constant for NADP photoreduction with little alteration of the light rate constant. In other words, the Hill reaction system was impaired somewhat in the light and dark reactions, whereas the NADP photoreduction with ascorbate and dye exhibited a change in only the dark rate-limiting step.

Stability of Activity—A serious problem encountered in attempting to determine the activity of chloroplast fragments was the rapid loss of the Hill activity during centrifugal fractionation of the fragments. The activity of the fragments was determined after fractionation of the sonically treated chloroplasts by successive centrifugations. The complete fractionation required about 7 hours from the time of homogenization of spinach leaves. Within a comparable time, the activity of untreated chloroplasts, stored at 0° in the dark, decreased gradually and was 50 to 70% of the original activity. On the other hand, sonically treated chloroplasts exhibited a rapid initial decrease of the activity to a level less than half of the original activity, within a few hours after sonic treatment, followed by a relatively slow decrease in activity with time. Becker et al. (5) stated that the Hill activity of chloroplast fragments was stable during storage, but they determined activity only at 7 and 24 hours after preparation of the washed chloroplasts.

A procedure which was found to be effective in retaining activity was the inclusion of methanol (15%) in the chloroplasts suspension throughout sonic treatment and fractionation. Milner et al. (10) observed a similar protective effect of methanol on the Hill activity of small chloroplast fragments.

Another problem encountered was the rapid decrease of the Hill activity of the chloroplast fragments during the time of reaction. This inactivation was especially marked with small chloroplast fragments. For example, the rate of ferriyanide Hill activity of the smallest fragments, S₁₇₅, decreased to about one-half of the initial velocity after 1 min. Removal of oxygen from the reaction system provided some protection against this inactivation. In fact, the rate of the Hill reaction with NADP was always higher under anaerobic than aerobic conditions. These instabilities of the Hill reaction system must be taken into account in any interpretation of the present results.

On the other hand, the photoreduction of NADP with ascorbate and DCI was quite stable during aging of the chloroplast preparation (cf. Reference 9). Some inactivation during the reaction was also observed for this photoreduction system with small chloroplast fragments, but it was much less marked than for the Hill reaction.

Sonic Treatment Medium—When chloroplasts were sonically disrupted for 10 min in 0.05 M phosphate (pH 7.8) containing 0.4 M sucrose and 0.01 M NaCl, which were used in previous experiments (8), the amount of the chloroplast fragments remaining in the supernatant solution after centrifugation at 144,000 X g for 30 min was too small to determine the activity of this fraction. The extent of fragmentation of the chloroplasts was found, however, to depend on the composition of the sonic treatment medium, especially on the concentration of phosphate. The relationship between phosphate concentration, pH 7.8, and chloroplast disruption caused by 10 min of sonic treatment is shown in Fig. 4. The extent of fragmentation of the chloroplasts was determined by measuring the distribution of chlorophyll among the centrifugal fractions. It is apparent that disruption of the chloroplasts was most marked in deionized water and decreased with increasing concentration of phosphate.

A similar but much diminished effect was observed with NaCl, whereas sucrose (0.4 M) had no significant effect on the fragmentation of the chloroplasts.
Activities of Chloroplast Fragments. I

The activities of chloroplast fragments were studied, focusing on their reaction with ascorbate and DCI. The chloroplasts were washed and then suspended in deionized water. They were divided into aliquots, each containing 27.5 mg of chlorophyll, and then sonically disrupted and fractionated by differential centrifugation.

**Figure 4:** Effect of phosphate concentration on fragmentation of chloroplasts. The chloroplasts were washed once with the sucrose, phosphate, NaCl solution, twice with deionized water, and finally suspended in deionized water. Six aliquots of the suspension, each containing 27.5 mg of chlorophyll, were made up to a final volume of 50 ml containing the indicated concentration of phosphate, pH 7.8. Each suspension was sonically disrupted for 10 min and fractionated by differential centrifugation.

It was also noted that the chloroplast fragments had a tendency to aggregate on standing; the extent of aggregation was again dependent upon the concentration of salt. It is assumed, therefore, that at least a part of the protective effect of phosphate on disruption of chloroplasts by sonic treatment is attributable to reaggregation of the fragments, which is a function of the salt concentration.

Unfortunately, the sonic treatment medium wherein extensive fragmentation of the chloroplasts occurred was rather unfavorable for preservation of activity. For example, chloroplasts sonically disrupted in deionized water lost Hill activity much more rapidly than chloroplasts sonically disrupted in the medium containing sucrose, NaCl, and phosphate. After various attempts to define a suitable medium which would allow for both extensive fragmentation and preservation of activity, a solution of 0.005 M phosphate (pH 7.8) containing 0.4 M sucrose and 0.01 M NaCl was chosen as the sonic treatment medium. The inactivation with time of the Hill activity of chloroplasts sonically treated in this medium was approximately the same as that of chloroplasts sonically treated in a medium containing 0.05 M phosphate. However, in the lower phosphate medium, fragmentation of chloroplasts was so efficient that more than 10% of the chloroplast fragments was found in the S175 fraction.

**Figure 5:** Hill activity of chloroplast fragments. All activities were determined in the presence of 2 x 10^{-3} M ammonium chloride. The activity of the untreated chloroplasts was determined at pH 7.6, whereas ferricyanide and NADP activity with the sonically treated chloroplasts was measured at pH 6. NADP activity was assayed anaerobically.

**Figure 6:** Photoreduction activities of chloroplast fragments with ascorbate and DCI. The reaction conditions were the same as in Fig. 3 with the exception that methyl red photoreduction was determined with red light illumination with a red glass filter which cut off below 650 nm and without the addition of ferredoxin and transhydrogenase. The activity of the P145 and S175 fractions was determined at pH 6. For NADP reduction, the following proteins were added: ferredoxin, 125 units (17); transhydrogenase, equivalent to 235 units of diaphorase activity (18); and plastocyanin, 42 mmoles.

**Activities of Fragments**—Typical activities for the Hill reaction and photoreductions with ascorbate and DCI for the various chloroplast fragments are presented in Figs. 5 and 6.

It is clear (Fig. 5) that the Hill activity did not decrease with decreasing size of the fragments in the presence of ammonium chloride, but rather exhibited a dependence on fragment size in a manner similar to that first described by Becker et al. (4, 5). The P5 fraction was whitish green in color and cloudy in appearance, and it was invariably less active than the smaller fragments. In most instances the highest activity was observed with the P145 fraction, but occasionally with larger fragment fractions. The smallest fragments, S175, were still active in the Hill reaction, but they were the least active of all the fractions with the exception of the P5 fraction.
It was found previously that the Hill activity with NADP, which disappeared during a few minutes of sonic treatment of chloroplasts, could be restored by the addition of plastocyanin (7). The restored activity showed essentially the same pattern of distribution among the various chloroplast fragments as the Hill reaction with ferricyanide (Fig. 5). Sonically treated chloroplasts were always less active than untreated chloroplasts, and the highest activity was obtained with fragments of medium size. It is assumed, therefore, that the rate-limiting step is probably related to the oxygen-evolving system (Photosystem 2).

The NADP photoreduction activity of the chloroplast fragments with ascorbate and DCI as electron donor behaved quite differently from the Hill activity. As mentioned previously, the activity with ascorbate and DCI was very stable during aging of the chloroplast fragments and the course of the reaction. Furthermore, following sonic treatment of the chloroplasts, NADP photoreduction activity with ascorbate and DCI increased in the presence of plastocyanin to a level higher than that of the untreated chloroplasts (Fig. 6). The distribution of this activity among the various chloroplast fragments was also quite different from that observed for the Hill reaction (Figs. 5 and 6). The activity of fragments larger than P145 was slightly less than that of the untreated chloroplasts, whereas the further decrease in the size of the fragments was associated with a remarkable increase of the activity. The small fragments required a several fold higher concentration of ferredoxin, plastocyanin, and transhydrogenase (NADP reductase) to observe maximal activity than did the sonically treated chloroplasts or larger fragments. The pH required for optimal activity with the fragments was slightly more acidic than with sonically disrupted chloroplasts. With saturating amounts of the three proteins and at a pH of 6, the P15 fraction always showed an activity greater than that of the sonically treated chloroplasts. Furthermore, the activity (550 to 1000 μmoles of NADP reduced per mg of chlorophyll per hour) of the smallest fragments, S175, was several times higher than that of the sonically treated chloroplasts.

Similar results were obtained for methyl red photoreduction with ascorbate and DCI as electron donor (Fig. 6). The smallest fragments exhibited an activity almost as high as the NADP photoreduction activity of the same preparation.

As indicated in Table I, a very high NADP photoreduction activity was also observed with the small fragments prepared by a second sonic treatment of larger fragments of lower activity.

**Table I**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>NADP photoreduction (μmoles/mg chlorophyll/hr)</th>
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<tbody>
<tr>
<td>A (S175)</td>
<td>880</td>
</tr>
<tr>
<td>B (P145)</td>
<td>194</td>
</tr>
<tr>
<td>C (P145 - P15)</td>
<td>340</td>
</tr>
<tr>
<td>D (P145 - S175)</td>
<td>950</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Photoreductions**—The photoreduction of NADP and methyl red with ascorbate and DCI is considered to be catalyzed only
by Photosystem 1 (8). These activities were increased by sonic treatment of the chloroplasts (Fig. 6). It was found, however, that fragments larger than P145 were less active than the sonically treated chloroplasts. The increased activity with sonically treated chloroplasts could, therefore, be attributed to the higher activity of the small chloroplast fragments. In a previous study, we reported that these activities underwent an initial rapid change during sonic treatment, which was dependent on pH and the presence of plastocyanin, but then remained constant and insensitive to ultrasound. However, in those earlier experiments, a high concentration of phosphate was used, and there was probably very little formation of the small chloroplast fragments which exhibit a very high activity.

The smallest chloroplast fragments, S175, deserve special attention because of their very high activity. One could suggest that the high activity of this fraction is due to the presence of an, as yet, unknown soluble factor which was extracted from the chloroplasts by sonication. This explanation is ruled out by the finding that larger fragments of low activity could be converted to small fragments of high activity by a second sonic treatment (Table I). This observation rules out a second possibility, suggested for detergent-treated chloroplasts (21), namely the preferential extraction by sonic treatment of a unique portion of the chloroplasts possessing a high capacity for NADP photoreduction with ascorbate and DCI but no Hill activity. In point of fact, the S175 fraction has the same pigment composition as untreated chloroplasts. The ratio of chlorophyll a to chlorophyll b is about 3, a value identical with that for untreated chloroplasts. This is in contrast to the small particles from detergent treatment where the ratio of chlorophyll a to chlorophyll b is about 6 (19, 21).

A similar change in activity, that is, an increase in NADP photoreduction by ascorbate and DCI with chloroplast fragments of decreasing size, was observed with detergent-treated chloroplasts (19, 21). Boardman and Anderson (21) reported that the smaller fragments from detergent-treated chloroplasts had a higher NADP photoreduction activity with ascorbate and dye and a lower Hill activity than the larger fragments. Vernon et al. (19) have shown that the small Triton-prepared chloroplast fragments photoreduced NADP with ascorbate and DCI at an extremely high rate (about 2000 μmoles per hour per mg of chlorophyll) but were devoid of Hill activity. In both reports (19, 21) a separation of the two photosystems of the chloroplasts was indicated since the ratio of chlorophyll a to chlorophyll b increased with decreasing size of the fragments.

The smallest fragments (S175) studied herein retained the requirement for ferredoxin, transhydrogenase, and plastocyanin (Table II) for NADP photoreduction. The Triton-prepared fragments exhibit the same requirements (19). In contrast, the photoreduction of NADP by ascorbate in a simple system, such as free chlorophyll or its derivatives, required either no proteins or only transhydrogenase (22, 23).

As shown in Table II, the photoreduction of NADP by the S175 fragments is dependent completely on ascorbate but only partially on DCI. The dye serves as an intermediate electron carrier between ascorbate and an electron-accepting site in the chloroplasts. Thus, it appears that sonic treatment resulted in an increased accessibility of the chloroplast fragments for electrons from ascorbate, probably via plastocyanin. It may well be that there is an inverse relationship between the dependence on DCI and plastocyanin concentration (20). The dependence on DCI would be more marked at low plastocyanin concentration. A similar relationship may well exist for the Triton-prepared chloroplast particles (19).

It is conceivable that the increased rate of NADP photoreduction with ascorbate (and DCI) is simply due to an increased availability of electrons to the chloroplasts from the electron donor system rather than a preferential extraction of Photosystem 1. With untreated chloroplasts, the photoreduction of NADP by the electron donor system is limited by a restricted availability of electrons. In fact, the rate of this photoreduction with untreated chloroplasts is usually lower than the rate of the Hill reaction. Thus, electrons provided by Photosystem 2 are more available for NADP photoreduction than electrons provided by ascorbate. Extensive sonic disruption of chloroplasts would relieve the system from this limitation, presumably by opening an oxidizing site for ascorbate via reduced plastocyanin. This explanation is supported by the observation that the increase in the rate of NADP photoreduction by sonic treatment was accompanied by a change in the dark rate-limiting step of the reaction (Fig. 3).

It is evident that the distribution of the NADP and methyl red photoreduction activities among the various fragments is not related in a simple fashion to their size. It appears that the retention of photoreduction activity may be related to the stability of Photosystem 1 to sonic treatment since the smallest fragments exhibited the highest photoreduction activity.

**Hill Activity**—Chloroplast fragments prepared by sonic disruption retain a significant Hill activity with ferricyanide. The values reported (Fig. 5) are considerably higher than those reported previously by other authors (3-5). For example, the rate of Hill activity with the smallest fragments, S175, was about 30% of the uncoupled rate of untreated chloroplasts (Fig. 5). These values must be regarded as minimum values because of the significant loss of Hill activity during the preparation of the fragments. In addition, the rate of the reaction decreased during the assay period.

Experiments designed to improve the reaction conditions revealed several noteworthy features of the Hill activity in sonically treated chloroplasts. First, a possible change in the site for reduction of ferricyanide by sonically treated chloroplasts was indicated by the lowered affinity of the photoreducing system for the oxidant. Secondly, the relationship between activity and light intensity disclosed an impairment in the photochemical step as well as in the dark rate-limiting step in sonically treated chloroplasts. These findings, together with the lowered activity of sonically treated chloroplasts as compared with untreated chloroplasts, suggest a significant destruction of Photosystem 2, the ferricyanide-reducing system, apart from fragmentation of the chloroplasts. They support the view of other authors (5, 6) and ourselves that there is no simple relationship between the activity and the size of the chloroplast fragments.

*This value may perhaps be slightly high. Izawa and Good (6) reported a rate of 800 μmoles of ferricyanide reduced per hour per mg of chlorophyll as the uncoupled rate of the Hill reaction by untreated chloroplasts. They used methylamine as the uncoupling agent, whereas we used ammonium chloride. Apparently methylamine is the better uncoupling agent. With their value, the activity of Fraction S175 is about 20% of the uncoupled rate of the untreated chloroplasts.*
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