PHOTOREDUCTION AND PHOTOOXIDATION OF CYTOCHROME C
BY SPINACH CHLOROPLAST PREPARATIONS

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The photoreduction of cytochrome c by spinach chloroplasts has been noted by several investigators (12, 20, 25). In a preliminary report, we have presented evidence that aqueous digitonin extracts of spinach chloroplasts contain a cytochrome c photo-oxidase (22) which appears to be similar to the photo-oxidase present in extracts of photosynthetic bacteria studied by Kamen and Vernon (13, 14, 32). The present paper describes the behavior toward cytochrome c of whole chloroplasts prepared in hypertonic salt solution, and outlines the experiments which led to the demonstration of their cytochrome c-photo-oxidase activity.

MATERIALS AND METHODS

CYTOCHROME: The cytochrome c used in these studies was a preparation from horse heart obtained from the Sigma Chemical Co. Reduced cytochrome c was prepared by the method of Margoliash (18), or, alternatively, solutions of oxidized cytochrome c were reduced by addition of stoichiometric amounts of Na2S2O4. In both cases, salts were removed by dialysis against H2O. There was no difference between the behavior of these 2 reduced cytochrome preparations in the various enzyme tests used. All cytochrome solutions were analyzed prior to use for both oxidized and reduced cytochrome c by measurement of optical density at 550 mµ before and after oxidation by ferricyanide and reduction by Na2S2O4 (24).

DIGITONIN: Digitonin was obtained from Fisher Scientific Co., from National Biochemical Corp., and from Nutritional Biochemicals Co. It was recrystallized from ethanol. Although the various samples used exhibited different solubility characteristics, they all gave similar results in the experiments here described.

CHLOROPLAST SUSPENSION: Spinach was bought at the local markets. Suspensions of washed chloroplasts were prepared by differential centrifugation of ground spinach leaves in 0.35 M NaCl according to the procedure described by Arnon et al. (2), with the modification that the NaCl solution was 10-4 M in disodium ethylenediamine tetraacetate (disodium Versenate), and the chloroplasts were washed twice.

Chlorophyll was determined by the method of Arnon (1). Chloroplast suspensions were made up in the NaCl-Verseen medium to contain about 1 mg of chlorophyll per ml and were stored in this form at 4°C. Precautions were taken to avoid unnecessary exposure to light, but no effort was made to exclude light completely. Examination of the suspension by light microscopy showed no detectable cell fragments or grana, but the apparently intact chloroplasts had an abnormal, vacuolated appearance.

PROCEDURE FOR ILLUMINATING SUSPENSION AND MEASURING REACTIONS: Measurement of light absorption was made with a Beckman DU spectrophotometer in cells of 1.00 cm light path, against water as a blank, with the slit width knob set at 0.04 mm. An increase in O.D. (optical density, i.e., log Io/I, where Io is incident light and I is emergent light) of 1 unit at 550 mµ corresponded to the reduction of 0.15 micromoles (µM) of ferricytochrome c in a volume of 3.0 ml. The samples were illuminated by a beam of white light focussed from above into the 2nd position of the cuvette carrier of the Beckman spectrophotometer. The portion of the cuvette which extended above the cuvette holder was shielded so that no scattered light from the light beam illuminating the reaction mixture could reach the photocell of the instrument. The intensity of the light at the surface of the liquid was about 40 ft-c. For the determination of O.D. the cell was pulled out of the light beam into the 1st position (i.e., in front of the photocell) for a period of 4 to 5 seconds, which sufficed to take a reading. The temperature was maintained at 20° ± 0.2°C.

RESULTS

PHOTOREDUCTION AND DARK OXIDATION OF CYTOCHROME C: Suspensions of washed chloroplasts, prepared as described above, caused a photoreduction of added cytochrome c in the light, and an oxidation of reduced cytochrome c in the dark. The photoreduction was conveniently demonstrated in dilute phosphate buffer of pH 7.0, containing about 0.06 µM of oxidized cytochrome c in a final volume of 3.0 ml. This solution was allowed to come to temperature equilibrium, the optical density at 550 mµ was measured, and then a suitable aliquot (e.g., 0.05 ml) of the chloroplast suspension was stirred into the reaction mixture under conditions of minimum illumination, without removing the cuvette from the instrument. A series of readings were taken in the dark at 1-minute intervals, to determine the contribution of the chloroplast suspension to the optical density at 550 mµ, and to ascertain whether any changes oc-
curved due to swelling or settling of the chloroplast suspension, or to any reduction of cytochrome c in the dark. No dark reduction of the cytochrome c was ever observed. Changes associated with swelling or settling were estimated to be no greater than 10% of the changes measured, provided the amount of chloroplasts employed was less than that corresponding to 0.1 mg chlorophyll.

A typical experiment illustrating the photoreduction and the dark oxidation of cytochrome c in the presence of 3 different amounts of chloroplast suspension is shown in figure 1. The photoreduction stopped before the cytochrome was completely reduced. When the light was switched off (point D on the curves), the dark oxidation began, as shown by the decrease in optical density. Within the concentration range of chlorophyll employed in this experiment, the initial velocity of the photoreduction was approximately proportional to the amount of chlorophyll added. As higher concentrations were used, the reaction became too rapid for accurate measurement, light became limiting, and difficulty with settling of the suspension was likely to be encountered.

In the course of the investigation, rate measurements of photoreduction and dark oxidation were carried out with several hundred different chloroplast preparations. Approximately fifty were made by the same procedure, as described in the preceding section. With freshly prepared chloroplasts, the rates of photoreduction ranged from 15 to 63 μM of cytochrome c reduced per hour per mg chlorophyll. The chloroplast suspensions lost their activity slowly over a period of several days storage at 0°C. During the first few hours of storage, however, some of the preparations actually gained activity for photoreduction of cytochrome, but this rise was not always observed.

The dark oxidase activity of different chloroplast suspensions fluctuated even more than the capacity for photoreduction, and was not clearly correlated with the photoreduction. The kinetics of the 2 reactions were quite different. The photoreduction was saturated by relatively low concentrations of cytochrome so that the initial reaction was almost linear with time, but the dark oxidase was more sensitive to the concentration of reduced cytochrome, showing kinetics which sometimes were almost second order with respect to cytochrome concentration. This was not always the case, however. In general, the initial rate of the dark oxidation, when measured as shown in the experiments of figure 1, was about one third or one fourth the initial rate of photoreduction. At times, it was only one tenth as great, and more rarely, it was as fast as the photoreduction. When the dark oxidase activity was relatively high, the cycle of change from almost completely reduced to almost completely oxidized cytochrome could be repeated by alternating appropriate periods of light and dark up to 10 times. The rates gradually diminished, since the chloroplasts gradually lost their activity under the experimental conditions. Since the dark oxidase lost activity more rapidly than the photoreduction on storage at 0°C, suspensions kept in the cold for 1 or 2 days exhibited a smaller ratio of dark oxidation to photoreduction than fresh suspensions.

**Effects of Inhibitors:** Antimycin A, cyanide, and azide had no appreciable effect on the photoreduc-

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**Fig. 1 (left)** Photoreduction and dark oxidation of cytochrome c. Reaction mixtures contained 40 μM of potassium phosphate buffer of pH 7.0, 0.06 μM of cytochrome c, and water to make a final volume of 3.0 ml. The lower, middle, and upper curves were obtained with chloroplasts containing 0.0044, 0.0176, and 0.0705 mg of chlorophyll, respectively. Light was turned on at time 0, turned off at arrow D, for each experiment.

**Fig. 2 (right)** Unmasking of photooxidase by digitonin. Reaction mixture contained 40 μM of phosphate buffer of pH 7.0, chloroplasts containing 0.1 mg of chlorophyll, 0.3 μM of KCN, and 0.056 μM of cytochrome c. L: light; D: dark. At the vertical arrow, 0.2 ml of aqueous 1% digitonin were added. O.D. readings corrected as described in text.
tion of cytochrome c, but the dark oxidase was inhibited almost completely by cyanide and by azide. Some representative data showing the effect of these 2 compounds are assembled in table I, which also shows that the photoreduction is inhibited by increasing the concentration of phosphate buffer. All the rate measurements listed in table I were carried out with the same chloroplast suspension, which had been stored for several hours. The effects observed were reproducible with other suspensions, except for the effect of phosphate on the dark oxidase. In the particular experiment shown in table I, the higher concentration of phosphate caused a moderate stimulation of the dark oxidase, but with other chloroplast preparations, the higher concentration of phosphate sometimes caused a small inhibition of the dark oxidase.

Table I also shows the percent of cytochrome in the reduced form when the steady-state level was reached, i.e., when the optical density at 550 m\(\mu\) showed no further change on continued illumination. As already mentioned, the photoreduction of cytochrome did not reach completion. The level of reduction of the cytochrome was calculated from the observed change in optical density and the known amount of cytochrome added, and this calculation was corroborated by addition of sodium hydrosulfite at the end of the experiments with measurement of the attendant increase in optical density which accompanied the complete reduction of the cytochrome.

In the experiments with added cyanide, precautions were required in the determination of the steady-state level. The oxidized form of cytochrome c forms a complex with cyanide, and this cytoferricytochrome c complex is not photoreduced by the chloroplasts. In this respect, the photoreduction of cytochrome c is completely analogous to the reduction of cytochrome c by succinate in the presence of preparations from animal tissues. Horecker and Kornberg (9) have studied the cytochrome c-cyanide complex in some detail, using the extent of reduction by succinate as a measure of the amount of uncomplexed cytochrome present. It is possible to use the photoreduction of cytochrome by chloroplasts in the same fashion.

**Table I**

**Effect of Phosphate, Azide and Cyanide on the Chloroplast-Cytochrome c Reactions**

<table>
<thead>
<tr>
<th>PHOSPHATE</th>
<th>OTHER ADDITIONS</th>
<th>PHOTOREDUCTION</th>
<th>DARK OXIDATION</th>
<th>STEADY STATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>(\mu)M</td>
<td>(\mu)M</td>
<td>(%)</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>...</td>
<td>9.1</td>
<td>1.9</td>
<td>90</td>
</tr>
<tr>
<td>200</td>
<td>...</td>
<td>4.4</td>
<td>2.5</td>
<td>51</td>
</tr>
<tr>
<td>200</td>
<td>0.3 KCN</td>
<td>4.1</td>
<td>0.3</td>
<td>79</td>
</tr>
<tr>
<td>200</td>
<td>0.3 NaN(_3)</td>
<td>3.9</td>
<td>1.1</td>
<td>70</td>
</tr>
<tr>
<td>200</td>
<td>15 NaN(_3)</td>
<td>4.4</td>
<td>0.2</td>
<td>71</td>
</tr>
</tbody>
</table>

* Initial rate, micromoles per hour per mg chlorophyll.

The reaction mixtures contained 0.036 \(\mu\)M of cytochrome c and chloroplasts containing 0.05 mg of chlorophyll, plus other additions as indicated, in a volume of 3 ml.

**Percent cytochrome c in reduced form.**

**Reduction by Succinate:** Brummond and Burris (4) have reported that the succinic dehydrogenase activity of green leaves is soluble and unstable. They measured this enzyme reaction by coupling it with N-methyl phenazine sulfate. In the present experiments, fresh chloroplast suspensions with added cyanide to inhibit the dark oxidase, were shown to catalyze the reduction of cytochrome c in the dark by...
added succinate. This activity was very unstable relative to the other activities measured, and disappeared on storage of the chloroplasts for several hours. A typical measurement with 0.3 μM of KCN and 10 μM of succinate under the other conditions described in the legend for figure 1 gave an O.D. increase at 550 μM of 0.070, equivalent to 0.01 μM cytochrome c reduced per minute per mg chlorophyll. This reduction of cytochrome by succinate was not inhibited by antimycin A or by the addition of digitonin. Thus, chloroplasts in the presence of digitonin, cyanide, and succinate, would reduce cytochrome c in the dark and oxidize it in the light, in direct contrast to the behavior of unsupplemented chloroplasts, which reduced cytochrome c in the light and oxidized it in the dark.

SOLUBILIZATION AND PROPERTIES OF CYTOCHROME c PHOTOOXIDASE: Digitonin has been used previously to prepare chlorophyll-lipid-protein complexes which are not sedimentable by ordinary centrifugation and have been called “chloroplastin” (15, 29, 30, 31, 33). The following experiments showed that such solutions contain the cytochrome c photooxidase activity. Twice washed chloroplasts from 200 g of spinach leaves were extracted at 0°C with 3 successive 10-ml portions of 1% digitonin, the 1st for 30 minutes, the 2nd for 40 minutes, the 3rd for 2.5 hours. Each successive extract was separated from the residue by centrifugation at 75,000 × G for 30 minutes. These extracts were labelled A, B, and C, in the order of their preparation. When such extracts were centrifuged at 30 minutes at 144,000 × G in a Spinco preparative ultracentrifuge, relatively trivial amounts of sediment were obtained, and the activity of the supernatant solution remained unchanged. It was possible, however, to draw off a turbid yellow supernatant from the top few millimeters,

just above the clear green boundary which had appeared during the centrifugation. This yellow supernatant was free from chlorophyll, and contained no trace of photooxidase activity. This implied that “chloroplastin” was necessary for the photooxidase activity. Nevertheless, the activity of the successive extracts was not proportional to their chlorophyll content, as shown by the data given in table II. Included in this table are the results of experiments showing that the photooxidase was completely inactivated by heat, and that the various heated extracts all contained an activator for the photooxidase.

The assay of cytochrome c photooxidase was based on the fact that cyanide inhibits the dark oxidase activity of the chloroplastin extracts, but not the photooxidase. Curve 1 of figure 3 shows the dark and light oxidation of ferrocytochrome c observed in the presence of cyanide. In the absence of cyanide (curve 2), the dark oxidation was more rapid, and the change in rate on illumination was not so striking. Examination of a large number of preparations showed that the ratio between the rate of the dark oxidation, measured in the absence of cyanide, to the rate of the photooxidation, measured in the presence of cyanide, varied considerably. The rate of oxidation of cytochrome c in the light in the absence of cyanide was either equal to or slightly greater than the rate in the light in the presence of cyanide. The data suggested that the 2 reactions were independent, at least in part, though they might have a common “bottleneck.”

Under the conditions used in the experiment shown in figure 3, the initial rate of photooxidation in the presence of cyanide was almost proportional to the amount of extract added, provided the concentration of reagents was kept constant and provided the rates were not faster than ΔO.D. = 0.020/min. (The figures given in table II were measured in this way.) This apparent proportionality was due to 2 opposing effects which tended to balance each other. The photooxidase activity of the digitonin extracts required 2 heat-labile components, present in the extracts. However, as larger amounts of chlorophyll were employed, light became more limiting, so the dependence of rate on the square of the concentration of extract was not evident except at very low concentrations. Cytochrome c photooxidase was strongly stimulated by phosphate and other salts, an effect which will be described in another paper.

The photooxidase was not affected by freezing and could be stored for months at −15°C. On storage at 4°C there was a progressive and somewhat variable inactivation which amounted to as much as 50% in 4 days. The activity was stable to dialysis against dilute phosphate buffer of pH 7.0. Both the dark oxidase and the photooxidase were heat-inactivated. There was no precipitate formed when digitonin extract B was heated, so this extract was generally used for testing effects of heat.

Table II
CHLOROPHYLL CONTENT AND PHOTOOXIDASE ACTIVITY OF SUCCESSIVE DIGITONIN EXTRACTS

<table>
<thead>
<tr>
<th>Extract</th>
<th>Heated Extract *</th>
<th>Chlorophyll ** mg/ml</th>
<th>Photooxidase Activity †</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A′</td>
<td>0.096</td>
<td>5.1</td>
</tr>
<tr>
<td>B</td>
<td>B′</td>
<td>0.216</td>
<td>3.5</td>
</tr>
<tr>
<td>C</td>
<td>C′</td>
<td>0.074</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Held at 90°C for 5 minutes. 0.2 ml used per reaction mixture.
** Chlorophyll content of unheated extract.
† Micrograms cytochrome c oxidized per hour per mg chlorophyll of the unheated extract. Assay mixtures contained 300 μM of phosphate buffer of pH 7.0, 0.03 μM of reduced cytochrome c and other additions as indicated, in a volume of 3 ml. The unheated extract was used in sufficient amount to give approximately 0.05 mg chlorophyll.
Curve 1 shows that there was no significant change in optical density when reduced cytochrome c was incubated with 0.2 ml of heated digitonin extract. The 1st 6 minutes of incubation were in the dark. Then the light was turned on at 6 minutes (broken line). At 28 minutes the light was turned off and 0.1 ml of unheated extract was added (arrow D). The change due to the dark oxidase was noted, and then the light was switched on at arrow L. The rate of photooxidation is seen to be faster than the activity displayed by 0.2 ml of unheated extract alone (curve 3). Curve 2 shows the activity of 0.1 ml of unheated extract. Curves 2 and 3 show that the rate of photooxidation with 0.2 ml of extract is twice as great as the rate with 0.1 ml of extract. Addition of 2 mg of digitonin had no effect on the reaction rates observed.

The heat instability of the photooxidase implied that the reaction was enzymatic. To corroborate this conclusion, 0.2 ml of a fresh acetone extract of the chloroplasts, containing an amount of chlorophyll comparable to that used in the photooxidase test, was added to the cytochrome c-phosphate buffer test system. No activity whatever was observed. Acetone in the quantity employed did not inhibit the photooxidase activity of the digitonin extracts.

The choice of pH 7.0 for the measurement of the photooxidase represented a compromise between the apparent optima for the photooxidase activity and for the cyanide inhibition of the dark oxidase. The latter was completely inhibited by cyanide (10^-4 M) at pH 7.5 and 8.0, but was only partially inhibited at lower pH values, possibly because of the volatilization of HCN. A pH of 6.3 appeared to be more nearly optimal for the photooxidase, giving a 20% increase in rate over that observed at pH 7.0. However, the effect of pH on the dark oxidase in the presence of cyanide was even greater, the rate at pH 6.3 being twice that at 7.0. The rates of the photooxidase at pH 7.5 and 8.0 were about two thirds and one third respectively of the rate at 7.0. In the range pH 5.3 to 5.9, turbidity developed and the photooxidase was inactivated.

Cytochrome c photooxidase requires the presence of oxygen, as shown in figure 5. Cytochrome c

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**Fig. 3 (left).** Demonstration and assay of cytochrome c photooxidase. Reaction mixtures contained 80 \( \mu M \) of phosphate buffer of pH 7.0, extract containing 0.04 mg of chlorophyll, and 0.06 \( \mu M \) of cytochrome c, of which 0.02 \( \mu M \) were in the reduced form, in a total volume of 3.0 ml. Curve 1: no KCN; curve 2: 0.3 \( \mu M \) of KCN. D: dark; L: light. Manipulations were carried out as described for the experiments of figure 1. O.D. readings corrected for optical density of extract and oxidized cytochrome c.

**Fig. 4 (right).** Photooxidase of dialyzed extract. Experiments were carried out with digitonin extract B, containing 0.18 mg of chlorophyll per ml, dialyzed for 16 hours at 4°C against 0.01 M phosphate buffer of pH 7.0. All reaction mixtures contained 40 \( \mu M \) of phosphate buffer of pH 7.0; 0.02 \( \mu M \) of reduced cytochrome c, 0.3 \( \mu M \) of KCN, and other additions as indicated, to a total volume of 3.0 ml. All reactions were started in the dark, and light was turned on at 6 minutes. Curve 1: 0.2 ml of extract B which had been held at 90°C for 5 minutes was added at time 0; at D, light was turned off, and 0.1 ml of unheated extract B was added; at L, light was turned on. Curve 2: 0.1 ml of unheated extract B added at time 0. Curve 3: 0.2 ml of unheated extract B added at time 0. All O.D. readings corrected for optical density of extract and oxidized cytochrome c.
photooxidase was not affected by added catalase or TPN, and was completely inactive toward 2,6-di- chlorobenzenone-indophenol. In the latter test, 0.05 \( \mu \text{M} \) of dye were used, and optical density readings were taken at 620 m\( \mu \). The dye was neither reduced nor oxidized either in the light or in the dark in the presence of the cytochrome c photooxidase preparations.

Discussion

Chloroplasts prepared by the method of Arnon et al (2) resemble those studied by Jagendorf (12) in that photoreduction of cytochrome c can readily be demonstrated in the absence of respiratory inhibitors. With the chloroplast suspensions studied by Rosenberg and Ducet (25) and by Mehler (20), it was necessary to inhibit the oxidase by cyanide in order to demonstrate that cytochrome c could function as a Hill reagent. McClendon (19) and Jagendorf and Wildman (11, 12) have described chloroplast preparations devoid of cytochrome oxidase, and are of the opinion that this enzyme activity represents a "contamination" of chloroplasts with other particulate matter from the leaf. On the other hand, Sissakian and his co-workers (27, 28) favor the view that cytochrome oxidase activity is indigenous to the chloroplasts. We have been impressed in the present experiments with the great variability of the (dark) cytochrome oxidase content of the various chloroplast preparations, and with the occasional very high values observed for the activity of this enzyme. Furthermore, on the one occasion when this was deliberately tested, the dark oxidase activity of the chloroplasts was enhanced by repeated washing with 0.35 M NaCl solution. The experiments of James and Das (10) have shown clearly that Krebs cycle enzymes with associated respiratory enzymes are separable from chloroplasts in a mitochondrial fraction. It is quite possible that the chloroplasts prepared according to Arnon et al (2) are contaminated with mitochondria to some extent, but it is unlikely that any such contamination represents a very high proportion of the bulk of the preparation. (We have not been able to see any evidence of contamination under the light microscope, and examination of the preparations with the electron microscope revealed only a small proportion of debris which did not appear to be of chloroplastic origin.) We do not regard the fact that chloroplasts can be prepared so as to be relatively free of cytochrome oxidase as evidence that the oxidase has been removed rather than inactivated. Since the cytochrome oxidase activity of the chloroplasts was sometimes quite high, the implication is that the cytochrome oxidase activity of the contaminating mitochondria must be very high indeed. James and Das (10) report that mitochondria from broad bean leaves have cytochrome c oxidase activity amounting to 560 \( \mu \) of \( O_2 \) per hour per mg of N. Values approaching this level were sometimes observed with the whole chloroplasts.

The above remarks refer to the dark cytochrome oxidase activity of the whole chloroplasts. For the cytochrome c photooxidase, there can be no question regarding its chloroplastic origin, since, as will be shown in a subsequent paper, a chlorophyll-lipid-protein complex is one of the components essential for the photooxidation reaction. The effect of digitonin on the photooxidase is regarded as an unmasking. When the chloroplasts are treated with digitonin they undergo extensive fragmentation, and lose their capacity to photoreduce cytochrome c. This permits their photooxidase activity to be demonstrated directly. The mechanism of the inhibitory effect of digitonin on the photoreducing capacity of the chloroplasts is of some interest. When plant mitochondria are treated with digitonin, their capacity for causing the dark oxidation of cytochrome c is enhanced (26). One might suppose that the photooxidase activity is enhanced by digitonin in a similar manner, but it is unlikely that the entire effect of digitonin on chloroplasts can be explained in this way. The behavior of the preparations suggests a severe inhibition of the photoreducing power of the system. It should be noted, however, that the action of the digitonin is gradual, and to some extent reversible. Chloroplast preparations which appear to have been completely

![Fig. 5. Dependence of photooxidase on \( O_2 \). Reaction mixtures contained 80 \( \mu \)M of phosphate buffer of pH 7.0, 0.3 \( \mu \)M of KCN, extract containing 0.021 mg of chlorophyll, and 0.108 \( \mu \)M of cytochrome c of which 0.038 \( \mu \)M were in the reduced form, in a total volume of 3.0 ml. O.D. readings corrected for chlorophyll and oxidized cytochrome. D: dark; L: light. Both reaction mixtures were illuminated with 2,000 ft-c outside of the Beckman spectrophotometer for carefully timed intervals. O.D. was measured by inserting the cuvette in the cell holder and quickly taking a reading. The anaerobic incubation was carried out in a cuvette fitted with a hollow stopper and side arm for evacuation. The chlorophyll-containing solution was added from the stopper after air had been removed by thorough evacuation.](image-url)
inhibited insofar as photoreduction of cytochrome c is concerned, can be partially reactivated by washing out the digitonin. When chloroplasts are extracted with aqueous digitonin, considerable green sediment always remains after centrifugation. Only a relatively small proportion of the total chlorophyll is solubilized to a point where it is not very readily sedimented at 144,000 × G. From chloroplasts suspended in aqueous digitonin one can in fact obtain, by suitable differential centrifugation, green fragments smaller than grana and capable of photophosphorylation as well as of photoreduction (5). The digitonin extracts described in the present paper do not yield such fragments because these have been removed by the high-speed centrifugation. These facts are of interest in connection with the recent report of Eversole and Wolken (8) who have described a number of photochemical activities in "chloroplastin" preparations.

Davenport, Hill and Whatley (6, 7) have described a protein factor present in leaf extracts necessary for the photoreduction of methemoglobin and cytochrome c by chloroplast suspensions prepared in a different way from those used in the present experiments. A possible explanation for the effect of digitonin in our experiments is that it causes the release or inhibition of this factor, which is presumably present in sufficient amount in the intact chloroplasts to permit photoreduction of cytochrome c without added activator. The digitonin may act partially in this manner, though it probably has a more deep-seated additional effect on the photolysis reaction, since the photoreduction of Hill reagents other than cytochrome c is also inhibited by digitonin treatment. The digitonin apparently inhibits the O2-evolving system. The possibility that the cytochrome c photooxidase is an artifact created by the digitonin has been carefully considered. We have already pointed out (22) that the cytochrome c photooxidase here described is different from the non-enzymatic photochemical reactions studied by Krasnovsky and his associates (16, 17). This difference is even more clearly brought out by the demonstration that the enzymatic photooxidase can be fractionated into 2 heat-labile components, one containing chlorophyll, and one not (21, 23).

We view the cytochrome c photooxidase as a component of the intact chloroplasts, partly because of the kinetic measurements which led us to suspect the presence of the photooxidase before we found a procedure for unmasking it. It is of interest that the data obtained with heavy oxygen by Brown and Good (3) seems entirely compatible with the presence of a cytochrome c photooxidase in their preparations. Their data with cyanide-poisoned chlorella suggests, in fact, that a cyanide-insensitive photooxidase is operating in these algae. Perhaps the photooxidase described in the present paper can account in part for that component of the oxygen absorption which does not appear to involve reduction of O2 to H2O2.

Summary

This paper describes the behavior toward cytochrome c of spinach chloroplasts prepared in a suspending medium of 0.35 M NaCl. The chloroplasts cause a photoreduction of ferricytochrome c, and a dark oxidation of ferrocytochrome c. They also contain a cytochrome c photooxidase, which can be clearly demonstrated after the photoreduction reaction has been inhibited with digitonin. The enzyme or enzyme complex causing the photooxidation of cytochrome c can be solubilized by treatment with digitonin. The cytochrome c photooxidase is not inhibited by cyanide and may thus be distinguished from the cyanide-sensitive dark oxidase.

The authors are indebted to Drs. Hans Gaffron and Norman Bishop for stimulating discussion.

Literature Cited

The preceding paper described evidence for the presence of a cytochrome c photooxidase activity in digitonin extracts prepared from washed spinach chloroplasts (3). The present paper describes how this photooxidase may be fractionated into 2 components, designated Factor 1 and Factor 2, both of which are necessary for enzyme activity. Factor 2 is soluble, and a procedure is described for its purification.

**Materials and Methods**

**Measurement of Cytochrome c Photooxidase:** The assay previously described (3) depended on the use of cyanide to inhibit the dark cytochrome oxidase.

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2. This investigation has been aided by grants from the American Cancer Society, from the National Science Foundation, and from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.
3. Present address: U.S. Salinity Laboratory, Riverside, California.

FRACTIONATION AND PURIFICATION OF CYTOCHROME C PHOTOOXIDASE OF SPINACH

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and measurement of the rate of photooxidation of cytochrome c by following the decrease in absorption at 550 m\(\mu\). This assay procedure was used during the experiments leading to the initial fractionation of the enzyme components. Protein was determined according to Lowry et al (2).

**Preparation of Digitonin Extracts:** The medium used to prepare the washed chloroplasts contained 0.35 M NaCl and 10-11 M disodium ethylenediaminetetraacetate. This is referred to as the NaCl-Versene solution.

Fresh washed spinach leaves were drained well, and stems were removed. Five 200-g lots of leaves were conveniently handled at one time. Each lot was chopped fine and transferred to a Waring blendor with an equal volume (200 ml) of ice-cold NaCl-Versene solution. The material was blended for 4 or 5 half-minute periods with the variac set at 90 v. In the intervals, the suspension was stirred to resuspend the solids. The homogenate was strained through 2 layers of...