Final Report

STUDIES ON THE HILL REACTION ACTIVITY OF SOLUBLE CHLOROPLAST EXTRACTS

Prepared for:

OFFICE OF RESEARCH GRANTS AND CONTRACTS
CODE SC
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
WASHINGTON, D.C. 20546

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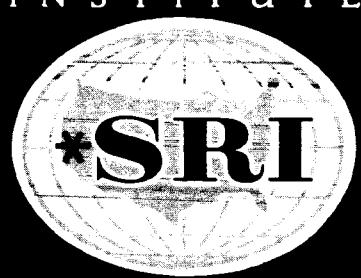
STANFORD RESEARCH INSTITUTE

MENLO PARK, CALIFORNIA



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ILLUSTRATIONS

[a]Eleven transparent overlays for chloroplast preparations and electrophoretic fractions are contained in the pocket of the rear cover.

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I INTRODUCTION

This study is concerned with the mechanisms and essential reactants of that part of the photosynthetic process in which oxygen is produced by photolysis of water. This is essentially the light absorbing reaction, which can be studied in vitro separately from the carbon dioxide fixing reactions. The photolysis of water and evolution of oxygen that occurs when chloroplasts or fragments of them are illuminated in the presence of a suitable electron acceptor is generally referred to as the Hill reaction.[1-4] Attempts to reduce the Hill reaction system to a nonparticulate state have generally resulted in loss of activity. Progress toward this goal would be useful in defining the minimum components and conditions required for photolytic activity, as well as in determining the details of the mechanisms of energy transfer. Recent experiments along these lines in our laboratories led to establishment of this project to study chloroplast extracts.

The long range plan of study includes analytical investigations to identify and characterize the components of the photoactive complex, and to determine the function of each; examination of the role of proteins, expecially enzymes, in the complex; studies on methods of preparing extracts of chloroplasts, and the stability of the preparations; determination of the participation and role of various cofactors; and application of spectroscopic and other physical methods to elucidation of the characteristics of the system.

II SUMMARY AND RECOMMENDATIONS

Carrier-free continuous electrophoresis has been applied to chloroplast fragments from common spinach, Spinacia oleracea. The electrophoretic fractions were tested as biochemical catalysts in the Hill reaction, the liberation of oxygen from water in the presence of light and ferricyanide.

Specific Hill reaction activity was sucessfully demonstrated after excursion through the Elphor FF, a commercial preparative electrophoresis instrument. The activity was greater for electrophoretic effluents than for unfractionated material. Maximum activity in the effluents did not correspond to the maximum chlorophyll concentrations, indicating either inhibition by certain components or some optimum proportion of catalytic cofactors. Differences is absorption spectra of fractions occurred in the ultraviolet region but were not significant in the visible region.

Novel methods to preserve the biochemical integrity of components were also studied so that extended experimentation could be performed with minimum loss of activity. Although digitonin facilitated the solubilization of the pigment-protein complex, some Hill reaction activity was lost. At the sacrifice of activity, however, fractionation of digitonin complexes occurred during Sephadex chromatography. DMSO was a useful solubilizing agent with no detrimental effect on Hill activity. Preservation of activity was obtained by adding colloids, such as normal and immune rabbit serum, gelatin, and dextran, to the solvent for chloroplast preparation.

It is recommended that future research should involve a study of the proteins in fractions separated electrophoretically or by chromatography on Sephadex or modified cellulose ion exchangers. The lipids and pigments should be extracted with organic solvents. The proteins should then be separated chromatographically and characterized by structure and amino acid composition.

III BACKGROUND

Chemical events in photosynthesis can be described by two major reactions. One is the light absorbing reaction; the other, the carbon dioxide fixing reaction. These two describe only the starting and ending points of a long chain of reactions, some of which are as yet unknown.

For instance, the carbon dioxide fixing reaction has been followed by using tracers and identifying the progress of the tagged reactions by radioautography. The work of Calvin and his group has been largely successful in following the main course of this reaction.

The light absorbing reaction can be studied separately from carbon dioxide fixation. Under those conditions, it is called the Hill reaction. It proceeds in the presence of an electron acceptor, which is usually a complex inorganic ion or a compound of the quinone type. These electron acceptors are reduced when illuminated in the presence of chloroplasts or chloroplast fragments, accompanied by photolysis of water and liberation of oxygen.

Most attempts to refine the photoactive preparation further, that is, to a nonparticulate state, have resulted in loss of the Hill reaction activity. Recent experiments in our laboratories showed that it is possible to prepare active extracts of whole chloroplasts or chloroplast fragments, using digitonin as a solubilizing agent. Chloroplasts were prepared according to the method of Arnon[5] and extracted with digitonin solution according to that of Nieman and Vennesland.[6,7] We have also applied these procedures to chloroplast fragments and are currently studying the retention of Hill reaction activity during storage of digitonin extracts under various conditions. The results indicate considerable stability.

The Hill reaction is the photolysis of water and the evolution of oxygen that occurs when chloroplasts or fragments of them are irradiated in the presence of an electron acceptor, e.g., ferricyanide, quinone,

or 2,6-dichlorophenol indophenol. When digitonin extracts were substituted for chloroplast fragments, moderately high rates of oxygen evolution were obtained only if cofactors, such as adenosine diphosphate, Mg^{++}, inorganic phosphate, ascorbic acid, flavin mononucleotide, and menadione (vitamin K_3), were added. In fact, no Hill reaction activity was obtained unless conditions were favorable for photosynthetic phosphorylation. However, we obtained no evidence for conversion of ADP to ATP. These results may be compared with some of those recently reported by Wolken,[8] who reported Hill reaction activity in chloroplastin prepared with digitonin.

Chloroplastin can be defined as a complex of proteins, lipids, phospholipids, chlorophylls, and carotenoids obtained from green plant tissues. The term "chloroplastin" was introduced in 1938 by Stoll and Wiedemann.[9] A description of the early work on suspensions and solutions was summarized by Rabinowitch.[10] Aqueous extracts were described by Lubimenko[11] and Loring and co workers;[12] these were prepared without the use of solubilizing agents. Euler and Hahn prepared chloroplastin by the Stoll method and reported the presence of ribonucleic acid.[13]

During the period 1938-1941, Smith made a rather thorough study of the solubilization of chloroplastin with detergents.[14-19] He was the first to use digitonin (a natural nonionic saponin gaining favor at that time with Tansley[20] and Wald[21,22]) as an extractant of photosensitive visual pigment-protein complexes, of sodium dodecyl sulfate,[23] and of the bile salts, sodium glycocholate and deoxycholate.[20] Smith's work has been repeated and extended by Ke and Clendenning[24] and by Wolken and his coworkers.[25-28]

Wolken is the only investigator to report Hill reaction activity for chloroplastin preparations, which were prepared with digitonin. Numerous workers has been unsuccessful in this respect, e.g., Ke and Clendenning,[24] French,[29] Chiba,[30] Kok,[31] and Vennesland and coworkers.[32,33] In fact, digitonin is described as an inhibitor in the oxygen-evolving process[33] and in photosynthetic phosphorylation.[32]

An explanation of this lack of success may involve exclusion of one of the cofactors that are necessary. Takashima described a chloroplyll-lipoprotein complex, but it was devoid of photochemical activity.[34] He noted that the carotenoids were excluded during the preparation of his complex.

That certain of the carotenoid pigments take part in the Hill reaction under some conditions has been shown by experiments of French and coworkers,[35,36] Bishop,[37-41] and Moss.[42] Much needs to be learned about their place in the sequence of steps making up the overall photolytic production of oxygen from water. Identifying the carotenoids that are essential or are capable of taking part is also not resolved. Some of the evidence on the ability of β-carotene to restore Hill reaction activity to chloroplast fragments after these were extracted with an organic solvent is conflicting. A number of cofactors are apparently involved; they need to be identified and their functions elucidated.

During recent years, digitonin and other solubilizing agents have been widely used for the preparation of various biologically active materials. Friend and Mayer used sodium deoxycholate to isolate a carotenoid-destroying enzyme.[43] Dowben and Koehler studied the effect of protein solubilization with Triton X-100.[44,45] According to Jirgensons,[46] the detergents Aerosol OT and HX form complexes with proteins and are much more efficient in unfolding macromolecules than are urea, guanidine salts, alcohol, and similar denaturing agents. Chiba used Duponol C and Span 80 for the solubilization of chloroplast proteins.[30] Ke and Clendenning extended the list of detergents, including Zephiran chloride, Tween 20, and purified saponin from several sources.[24] However, the most prolific group is with Vennesland at the University of Chicago. Vennesland and her coworkers have employed digitonin to fragment chloroplasts and have studied fractions for specific enzyme activity.[6,7,32,33,41,47,48,49,51]

Nieman and Vennesland characterized the enzyme cytochrome c photooxidase. This enzyme was botained from chloroplastin, which was

fractionated by ethanol precipitation[6] or by differential centrifugation.[33] No Hill reaction activity was evident, and it was presumed that digitonin inhibited oxygen liberation. According to Koukol,[32] Nakamura,[49] and Chow,[48] the catalysts involved in photosynthetic phosphorylation are particulate in nature. However, photophosphorylation suffered 50% inhibition in 0.03% digitonin solution and was completely inhibited by digitonin concentrations greater than 0.1%. Recently, Ardao and Vennesland observed chlorophyllase activity associated with chloroplastin.[47] The Koukol method[32] for preparing digitonin fragments was used by Krall and Purvis in their study of nucleotide acceptor specificity of photophosphorylation.[50]

We found that the visible absorption spectrum of chloroplastin solutions had a red peak with a maximum at 675 mμ, in agreement with spectra reported in the literature.[14,30] In addition, there was strong absorption in the 450 to 480 mμ region characteristic of the carotenoid components. The solutions also exhibited brilliant red fluorescence when irradiated with 365 mμ light. One of the more interesting experiments we performed with chloroplastin was column chromatography on Sephadex. The migration of the green band was easily followed and its fluorescence was demonstrated during chromatography on the column. We did not, however, determine Hill activity following chromatography.

IV EXPERIMENTAL

A. Chloroplast Preparation, Stability, and Storage

1. Typical Chloroplast Preparation

Chloroplasts and fragments were prepared from fresh spinach* The leaves were chilled, washed in distilled water, and deveined. They were then cut into small pieces and ground with either a mortar and pestle or minced in a Waring blendor in a cold solution (4°C) of 0.1 M KCl, 0.5 M sucrose, and 0.5 M phosphate buffer (pH 6.6). The leaves were either illuminated or kept in the dark before grinding. The sucrose-phosphate buffer was used at the rate of 1 ml/g of leaf tissue. The leaves were ground only enough to liquify the preparation and then strained through four layers of cheesecloth and one layer of glass wool.

The filtered suspension was centrifuged for 5 min at 2000 rpm (480 x g) to remove large cell debris, then for 15 min at 3000 rpm (1085 x g) to collect whole chloroplasts, and finally for 15 min at 15,000 rpm (27,000 x g) in a Servall centrifuge to collect chloroplast fragments. The pellets of chloroplasts or fragments were resuspended in sucrose-phosphate buffer, using a glass homogenizer. The sample preparation was all done under green light.[99] Samples not used immediately for Hill reaction assays were frozen in 3-ml aliquots and stored in the dark in a freezer (-40°C).

In recent experiments, a Tris buffer (0.025 M Tris [hydroxymethyl] amino methane, adjusted to pH 7.0 with HCl) was used because it was directly applicable to preparative electrophoretic separations.

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*Purchased at a local market.

2. Solutions containing Digitonin, Dimethyl Sulfoxide, and Caffeine.

Preparations were made as described above, through the first centrifugation at 2,000 rpm. The supernatant was centrifuged at 15,000 rpm, and the pellet was resuspended in 30 ml of sucrose-phosphate buffer with various amounts of digitonin added. The homogenized mixture was allowed to stand in the cold for 15 min and was then centrifuged at 3,000 rpm for 10 min. The supernatant (S_1) was separated from the pellet (P_1), and a portion was centrifuged at 15,000 rpm for 15 min. The S_2 supernatant was separated from the P_2 pellet, and a portion was again centrifuged at 15,000 rpm for 60 min to prepare pellet P_3 and supernatant S_3. The pellets were resuspended in sucrose-phosphate buffer for Hill reaction measurement.

Dimethyl sulfoxide and caffeine (both as reagent grade chemicals) were substituted for sucrose in the typical buffer solution used throughout the extraction and resuspension of chloroplast preparations. The DMSO was present either as a 0.4 M or 10% solution. In the case of caffeine, a 1% solution was employed.

3. Insonation

Whole chloroplasts were prepared from 100 g of spinach leaves. Pellets obtained by low speed centrifugation were resuspended in 50 ml of Tris-HCl buffer (pH 6.9) and sonified in a Raytheon 10-kc sonifier modified to achieve a 250-watt output. The chloroplasts were sonified for brief intervals at maximum power for 4 min.

4. Immunochemistry

The crude supernatant solution from a typical chloroplast fragment preparation was dialyzed overnight against 4 liters of distilled water. The experiment was carried out in a cold room at 4°C but, because of heat generated by a magnetic stirrer, the temperature of the solution rose to 30°C during the dialysis.

The dialyzed material was concentrated by lyophilization; 1.1 g was recovered from 150 g of spinach leaf tissue. A 250-mg portion of the lyophilized material was resuspended in 20 ml of Tris buffer, and the suspension was centrifuged for 15 min at 15,000 rpm. When the clear yellow solution was used in Hill reaction experiments, it had no effect on the photolytic activity of typical chloroplast fragments.

The dialyzed, lyophilized material was suspended in a neutral buffer (2 mg/ml Bacto-hemagglutination buffer) and sterilized by Millipore filtration. One part of this solution was mixed with an equal volume of Freund's complete adjuvant. The antigen solution (1 ml, containing 1 mg of lyophilized material) was injected intramuscularly into each of three New Zealand white rabbits every other day for the first week of immunization and then, as an antigen solution without the adjuvant, for the next three to four weeks on the same schedule.

Rabbits were bled one week after the final injection. The serum was separated from the clotted blood and preserved frozen in sterile serum bottles.

B. Centrifugal Fractionation

A Beckman/Spinco Preparative Ultracentrifuge (Model L-2) was used to fractionate the chloroplast preparation after sonication. The supernatant suspension was centrifuged at 20,000 x g for 15 min and a pellet, P_{0-20}, collected. The resulting supernatant suspension was then centrifuged an additional 15 min at 50,000 x g. The pellet was resuspended as fraction P_{20-50}. The supernatant fraction S_{50} was still green. Various spectrophotometric, Hill reaction, and chlorophyll determinations were made on these fractions.

C. Electrophoretic Fractionation

Preparative electrophoresis was performed in an Elphor FF, a carrier-free continuous electrophoretic separator. The instrument was designed by Hannig[52] and is distributed in the United States by Brinkmann Instruments, Inc., Westbury, New York 11590.

In contrast to curtain electrophoresis at 30 watts, a chloroplast fragment preparation can be studied easily on a preparative scale and under high power conditions at about 300 watts in the Elphor FF. The Elphor FF has no carrier; it is essentially two air-cooled glass plates about 50-cm square, separated by a 0.5-mm space through which the electrolyte passes as a film. The instrument was run at a constant current and observed under dim green light. Forty-eight fractions divide the effluent at centimeter intervals, the fraction numbers increasing from left (anode) to right (cathode). The red pilot lights on the instrument were masked to decrease their intensity.

Concentrated Tris buffer 0.075 M, pH 7.0, was used in the electrode chambers. The electrolyte passing through the chamber was Tris 0.025 M, pH 7.05. The buffer flow rate through the separating chamber was 363 ml effluent per hr (electrolyte peristaltic pump). The dosing rate was 10.5 ml per hr (sample peristaltic pump setting). The inlet port was 34-35. With these flow rates and electrolyte concentrations, the power settings were 160 ma and 1900 volts (approximately 304 watts). The temperature control was set at 4°C. A plastic bag containing crushed ice was placed over the plastic tubing leading from the dosing pump into the chamber.

The chloroplast fragment sample was prepared as follows for dosing into the Elphor FF. The supernatant liquid containing chloroplast fragments (P_2) was centrifuged for 15 min at 15,000 rpm (27,000 x g) in a Servall centrifuge. The high-speed pellets were resuspended in 20.0 ml of Tris buffer and homogenized with a hand homogenizer. The solution was then centrifuged for 10 min at full speed in a clinical centrifuge. The pellet was discarded and a portion of the suspension of P_2 was used in the high voltage electrophoresis unit. Figure 1, appended to the report, is a photograph of the Elphor FF, showing a chloroplast electrophoretic separation.

The chloroplast fragment sample (P_2) was subjected to approximately 38 v/cm during about 18 min in the separation chamber. Approximately 90 min was required for the electrophoretic separation.

In experiments involving digitonin extraction, the pellets (P_1) from slow speed centrifugation were resuspended in 20 ml Tris buffer containing 1% digitonin, allowed to stand for 15 min, and centrifuged for 10 min at 1000 x g The pellets were discarded but supernatant solution was then centrifuged in a Servall centrifuge for 15 min at 15,000 rpm (27,000 x g) to recover larger digitonin/chloroplast fragments (PD_2). The supernatant solution, containing very small digitonin/chloroplast fragments, was recentrifuged for 1 hr at 27,000 x g, and the pellets (PD_3) were resuspended in 5.0 ml of Tris buffer. The larger fragments (PD_2) from the 15-min centrifugation were also resuspended in Tris buffer and prepared for the Elphor FF as described for the P_2 fragments.

The lyophilized material used as the antigen mixture for rabbit immunization was also analyzed by electrophoresis in the Elphor FF, using a Tris buffer (pH 6.9). The inlet port was 34-35, dosing dial setting, 3.0; buffer dial setting, 2.5; residence time in the chamber, 41.7 min; 130 ma; 1500 volts. The effluent fractions from the Elphor were examined in a spectrophotometer at 260 mμ and 280 mμ. The material came off in fractions 22 through 36, showing that most of it was acidic at pH 6.9. A faint yellow color was visible in fractions 25 through 30. It is probable that a fraction of this soluble material is related to the Fraction I of Singer,[53,54] Wildman,[55-57] and others.[58-63]

D. Measurement of Hill Reaction Activity

Hill reaction activity of the fractions was determined by the usual manometric methods for oxygen evolution[64] in an Aminco Warburg apparatus or a constant volume refrigerated respirometer. All manipulations, before the exposure to red light during the photolysis reaction, were performed under dim green light. The temperature was maintained at 14.4 ± 0.1° C during runs. The conical Warburg vessels were illuminated with red light from below by a circular, five-tube neon bulb. The light intensity was approximately 9,000 lux. The 3-ml reaction volume in each Warburg flask consisted of 1.0 ml P_2, containing about 0.25-1.6 mg chlorophyll, 0.8 ml Tris buffer (pH 7.05), and 1 ml of Hill reaction

oxidant (0.01 mg potassium ferricyanide). There was 0.2 ml of 10% KOH in the center wells. Vessels and lines were flushed with prepurified nitrogen. Evolution of O_2 in each Warburg manometer was corrected against a thermobarometer.

The oxygen evolution rate was calculated as:

$$Q_{O_2}^{Chl} = \frac{\mu l\ O_2/min \times 60}{mg\ chlorophyll}$$

To measure oxygen by the polarographic electrode method, a Beckman oxygen sensor (39065) and adapter box (96260) were used in conjunction with a Model 76 Beckman expanded scale pH meter. After the equipment was set up, several minor adjustments had to be made to adapt it to use with a potentiometric recorder. The shorting strap across the recorder output on the pH meter was replaced with a resistor. Using a 1-mv recorder (Servo/Riter Model PSR, Texas Instruments, Inc.) with an attenuation path on the input circuit, a 49.5-ohm resistor, ± 1%, $\frac{1}{4}$ watt, gave a 10-mv recorder sensitivity. The recorder span was adjusted to read 75 μl of oxygen evolved per chart division. (Note: the technical assistance of Franklin M. Church during the installation of the new instrumentation is gratefully acknowledged.)

Experiments were conducted to compare the accuracy of the results obtained using the polarographic electrode with the Warburg respirometer. The oxygen-sensing polarographic electrode was calibrated in air using the X10 attenuation and the 100% scale on the oxygen adapter box. This gave a reading of 21% partial pressure of oxygen in air on the pH meter; the recorder was adjusted to read accordingly.

The electrode was inserted in a modified Warburg flask. An additional side tube was attached to the flask to facilitate flushing with prepurified nitrogen to obtain an anaerobic base line. The flask contained 1.0 ml of chloroplast fragment suspension in the side arm, 1.0 ml of 0.01 M potassium ferricyanide solution and 0.8 ml of 0.025 M Tris

buffer (pH 7.0) in the main portion of the flask, and 0.2 ml of 10% potassium hydroxide solution in the center well.

The flask was immersed in the constant temperature water bath (14.4 ± 0.1°C) of the Warburg apparatus (Model 5-134, American Instrument, Inc.) and shaken while being flushed with prepurified nitrogen. After approximately 20 min, a steady baseline was obtained on the recorder. The solutions were then mixed and allowed to stabilize in the dark for a few more minutes before the light was turned on to drive the photochemical reaction. Comparable flasks, including a thermobarometer, were run using standard manometric methods. The results by both methods compared favorably. In one run, the microliters of oxygen evolved per minute were 4.43 (manometric) and 4.5 (polarographic). In another experiment, they were 5.94 and 6.3, respectively.

E. Spectrophotometric Analyses

A Cary spectrophotometer (Model 14) was used to examine the spectra of the Elphor FF fractions in an aqueous solution. The P_{20-50} and S_{50} samples were also analyzed. For the more opaque solutions, a 1:100 dilution was made to get the complete spectrum on the chart; the spectra were scanned from 700 mμ to 200 mμ.

Chlorophyll determinations were made using the method of Arnon.[103] A 0.5-ml sample was diluted to 25 ml in 80% aqueous acetone. Absorbancy was measured at 645, 652, and 663 mμ. The chlorophyll concentrations were calculated from the formula:

$$\frac{O.D._{652} \times 1000}{34.5} \times \frac{25}{1000 \times 0.5} = \text{mg/ml}$$

V RESULTS AND DISCUSSION

A. Preparation, Storage, and Stability

One of the major problems in the study of the Hill reaction is the preservation of photosynthetic activity; this activity of plants is contained in the chloroplasts. During the isolation of these structures, the leave tissues are fragmented and barriers are destroyed that normally preserve the biochemical integrity of all the components. The Hill reaction enzyme system becomes rapidly and irreversibly inactivated in this crude milieux unless it is kept cold and protected from atmospheric oxygen.

In attempting research on this labile biochemical system, an initial objective was the preparation of chloroplasts and fragments under conditions that would give the highest initial activity. A corollary of this objective was the search for methods of preserving the Hill reaction activity so that extended experimentation could be performed with minimum loss of activity.

Illumination and alternative methods of grinding appeared to have relatively little effect on Hill reaction activity. The chloroplasts kept better than the fragments while stored frozen. Washing with large volumes of buffer did not appreciably affect the activity. In general, the preservation of activity in stored frozen samples was quite good. Storage results are summarized in Table I.

In the Background section, there is a discussion on the use of digitonin as a solubilizing agent for the preparation of what has been called "chloroplastin." Preliminary studies before this project was established indicated that digitonin solutions might well facilitate the solution of protein-pigment complexes and, hopefully, those demonstrating Hill reaction activity.

The experiments summarized in Table II suggested that the presence of digitonin might diminish the Hill reaction activity, especially in the later supernatant fractions.

Table I

EFFECT OF PREPARATIVE METHODS AND STORAGE ON HILL REACTION ACTIVITY

Experiment No.	Method of Grinding	Illumination	Preparation	$Q_{O_2}^{Chl}$ Days in Storage								
				0	½	1	4	7	13	24	29	49
A	Mortar	Yes	Chloroplasts	936								
			Fragments	1361								
B	Blendor	Yes	Chloroplasts	880								
			Fragments	818								
C	Mortar[a]	No	Chloroplasts	1064		829[b]						
			Fragments	820		469[b]						
D	Mortar[a]	No	Chloroplasts	661		700		600				
			Fragments	591		620		500				
E	Mortar	No	Chloroplasts		1410						1300	833
F	Mortar	No	Chloroplasts	900								
			Fragments	600								
G	Blendor	No	Chloroplasts	863			666		783			
			Fragments	1091					545	600		

[a] Washed with large volume of buffer after grinding.

[b] Stored in cold, not frozen.

Table II

EFFECT OF DIGITONIN CONCENTRATION ON HILL REACTION ACTIVITY

Digitonin Concentration (%)	$Q_{O_2}^{Chl}$					
	P_1	P_2	P_3	S_1	S_2	S_3
2	395	882	---	240	---	---
1	375	472	369	300	343	---
1	960	555	274	106	143	125
1	291	350	392	292	111	---
0.5	643	466	263	167	200	136
0.5	831	1181	---	260	171	---
1	584	1083	440	142	133	---

Attempts were made to improve the activity by Sephadex chromatography and by dialysis. The Sephadex columns were prepared with different grades suspended in 0.1 M KCl and 0.5 M sucrose. The column was developed with 0.5 M phosphate buffer and 0.5 M sucrose, pH 6.6.

At room temperature on Sephadex G-25, P_3S_3 formed two bands, one of which was eluted. $P_1P_3S_3$ together formed three bands, the first two of which were eluted. On Sephadex G-50, $P_1P_3S_3$ together formed four bands, of which the first two were easily eluted, the other two not at all. On Sephadex G-100, flow rates were very fast, and no separations were achieved. Unfortunately, all of the fractions eluted from Sephadex columns contained digitonin. At the low temperature of a cold room ($4^{\circ}C$), the solvents were too viscous to provide useful flow rates.

Two chloroplast preparations were prepared with 1% digitonin and used to investigate the effect of dialysis. In each case, after solubilization with digitonin solution, half of the sample was dialyzed in the cold against 3 liters of 0.5 M phosphate buffer, pH 6.6, and was stirred mechanically during the dialysis. The other half of the sample

was kept as a control at the same temperature. Both dialyzed and control samples were fractionated into pellet and supernatant fractions. The results of Hill reaction measurements on these fractions are shown in Table III.

Dialysis for 4 hr did not effectively remove enough of the digitonin, while dialysis for 19 hr removed most but not all. Dialysis had a beneficial effect in some cases, although not consistently. The loss of activity in controls during storage at 4°C discouraged the use of extended dialysis time.

Table III

EFFECT OF DIALYSIS ON HILL REACTION ACTIVITY OF DIGITONIN EXTRACTS

Preparation	$Q_{O_2}^{Chl}$						
	Whole Extract	P_1	P_2	P_3	S_1	S_2	S_3
Number 1							
Before dialysis	180						
19-hr dialysis	---*	---	165	270	---	390	---
19-hr control	90	---	180	---	150	109	---
Number 2							
Before dialysis	231		P_2 —— P_3				
4-hr dialysis	240	---	—250 —		---		---
4-hr control	90	---	156		150		---

* --- Indicates negligible activity

A new preparation of chloroplasts and fragments was made with 1% digitonin. Hill reaction activity was measured as soon as practical after preparation, again after one and two periods of incubation in the

cold, and finally after four days frozen storage. As seen by the results in Table IV, the main effect of digitonin is prompt, with very little further change during incubation or storage.

Table IV

EFFECT OF PERIOD OF EXPOSURE TO DIGITONIN ON HILL REACTION ACTIVITY

Preparation	Digitonin	$Q_{O_2}^{Chl}$			
		45 min	3.5 hr	7 hr	4 days storage
Chloroplasts	Yes	495	429	476	316
	No	1207	1013	901	896
		45 min	3 hr 10 min	6 hr 40 min	4 days storage
Fragments	Yes	469	444	480	230
	No	1216	800	816	1043

A number of preparations of both whole and fragmented chloroplasts were quick-frozen and lyophilized. Results of Hill reaction studies on these are given in Table V.

The effect of lyophilization seemed to be quite erratic and especially detrimental to the preparations of fragments. As seen with preparation F, it was possible to obtain a highly active lyophilized preparation. A limited storage study was performed on a lyophilized chloroplast preparation. Hill reaction activity was measured on a portion of the sample before the storage period. After one month, it was again measured immediately and at later intervals after thawing and resuspending the products. The results are shown in Table VI. Obviously, much less activity was lost by freezing than by lyophilizing. Apparently, considerable care is required in the freezing and dehydrating steps to preserve the Hill reaction activity.

Table V

EFFECT OF LYOPHILIZATION ON HILL REACTION ACTIVITY

Preparation	$Q_{O_2}^{Chl}$	
	Fresh Preparation	After Lyophilizing
A. Chloroplasts		600
B. Chloroplasts		618
Fragments		290
C. Chloroplasts		570
D. Chloroplasts	1143	470
Fragments		---
E. Chloroplasts	1014	451
Fragments	882	345
F. Chloroplasts		1105
Fragments		---

Table VI

COMPARISON OF THE EFFECT OF FREEZING AND LYOPHILIZATION ON THE HILL REACTION ACTIVITY

Chloroplast Preparation and Storage Time	$Q_{O_2}^{Chl}$			
		After storage		
	Initial	0	3 hr	5.5 hr
A. Frozen: 2.5 months	863	811	486	405
B. Lyophilized: 1 month	833	281	290	180

The difficulties encountered in the Sephadex chromatography of digitonin-solubilized chloroplast preparations prompted us to search for other substances that might have a protective effect and serve as an adequate substitute for the typical sucrose-phosphate buffer. Dimethyl sulfoxide (DMSO), which has been described as a useful nonaqueous solvent for many proteins [65,66] yields solutions without marked changes in the biological properties of the proteins. DMSO has unique solubility properties and is miscible in both polar and nonpolar solvents. When a 0.4 M solution showed no inhibitory effect on the Hill reaction activity of a chloroplast preparation, the concentration of DMSO was increased to 10% wt/vol. The results of these studies are included in Table VII.

DMSO may become a useful solubilizing agent. Within experimental error, the Hill reaction activity of solutions containing it was unchanged or slightly enhanced. Future studies should include its effect on the storage stability of chloroplast solutions kept at 4°C and at -40°C, its effectiveness in increasing digitonin solubility in aqueous solutions, and its potentiation or attenuation of energy transfer in the Hill reaction system.

Table VII

EFFECT OF DIMETHYL SULFOXIDE (DMSO) AND CAFFEINE ON HILL REACTION ACTIVITY

Solvent Systems	$Q_{O_2}^{Chl}$	
	Chloroplasts	Fragments
0.4 M DMSO	460	383
Control	551	439
10% DMSO	611	520
Control	551	611
1% Caffeine	387	352
Control	487	851

The recent paper by Fredericks and Jagendorf[67] described both the preservation of Hill reaction activity and the marked stimulation of some photosynthetic systems by high molecular weight polymers, such as Carbowax and dextran. The earlier work of Clendenning and Brown was cited.[68] Clendenning advanced the thesis that the Carbowax compensated for the inactivation of chloroplasts by leaf tannins released into intimate mixture with the Hill reaction components. The Hill reaction rates were inversely related to the tannin content. Furthermore, it was impossible to regain Hill reaction activity once it was lost as a consequence of tannin-protein precipitation.

On another project, we have been using tannic acid as a quantitative protein precipitant. The proteins were recovered unchanged by applying the novel caffeine method of Mejbaum-Katzenellenbogen and coworkers.[69-71] Although spinach has a negligible tannin content, we tested the effect of 1% caffeine content on the Hill reaction activity of a typical spinach chloroplast preparation with the results shown in Table VII.

Although there was a decrease in the Hill reaction activity of both whole chloroplasts and fragments, we do not consider 1% caffeine as a photosynthetic inhibitor. Stimulation of Hill reaction activity was reported by MacDowall[72] for another class of alkaloids, e.g., brucine and strychnine. Caffeine should be tested as a Hill reaction stimulator, using a photosynthetic system high in tannin content. In addition, the enhancement of Hill reaction activity by strychnine should be confirmed. Information of this nature would be helpful in extending the biological half-life of the Hill reaction system.

In search for highly active chloroplast preparations, we turned to differential centrifugation so effectively demonstrated by Gross, Becker, and Shefner[73-76] as an ideal method for separating chloroplast fragments. In addition, we used their method of disrupting the chloroplasts by insonation. The results of two experiments are summarized in Table VIII.

Table VIII

EFFECT OF INSONATION AND DIFFERENTIAL CENTRIFUGATION ON HILL REACTION ACTIVITY

Fraction	Expt. 1		Expt. 2	
	Chl		Chl	
	Q_{O_2}	mg/ml	Q_{O_2}	mg/ml
Nonsonified	419	0.430	---	---
Sonified	---	---	250	0.613
CF_{1-20}	336	1.18	560	0.948
$CF_{20}S$	466	0.193	---	---
CF_{20-50}	---	---	437	0.906
$CF_{50}S$	---	---	299	0.121

The data suggest that the smaller particles in the supernatant solution have higher Hill reaction activity than the larger particles sedimented at 20,000 x g. We have previously reported that the $Q_{O_2}^{Chl}$ varies as an inverse function of the chlorophyll concentration during the Hill reaction run. Thus, the low activity of CF_{1-20} may result from the use of two- to five-fold amounts of chlorophyll. The activity of CF_{1-20} was almost equal to that of CF_{20-50}, according to the results of Experiment 2. The crude mixture of sonified chloroplast fragments, as well as the supernatant solution of CF_{20-50}, showed less activity. Under conditions previously used to prepare chloroplast fragments, there would be low concentrations of chlorophyll and insignificant Hill reaction activity in supernatant solutions. In general, these results confirm the report of Becker, Shefner, and Gross that sonic disruption of chloroplasts facilitates fractionation into particles whose activity varies with sedimentation rate.

Gross and coworkers had observed the greatest Hill reaction activity in the CF_{20-50} fraction. In subsequent experiments, we have

called it the P_{20-50} fraction. Its use in electrophoretic studies will be described below.

During the preparation of chloroplast fragments, the soluble fraction in the supernatant solution is discarded as the components are not required for the photolytic processes of photosynthesis. An experiment was performed to isolate all the nondialyzable components in the soluble fraction for the production of antisera.

The rationale for studying soluble components is based on conclusions of Arnon and Whatley[77] and numerous reports describing the successful inhibition of enzymes by specific antisera, i.e., use of antienzymes.[55,78-80] After confirming the importance of chloride ion as a soluble cofactor of the Hill reaction, Arnon and Whatley[77] sought other "soluble" cofactors. Their results suggested an unavoidable large degree of deterioration during the relatively long period of high speed centrifugation when the cytoplasmic fluid and chloroplast fragments were combined.

Without exception, all available information on the nature of the components active in the Hill reaction emphasizes their particulate structure. The specific Hill reaction enzymes are attached to particles and are not unbound, soluble substances. In contrast, many enzymes involved in the "dark" reactions have been characterized as typical, soluble proteins. (See discussions by Losada and Arnon.[81]) It is our hypothesis that numerous degradative enzymes can be found among the soluble components, e.g., phosphatases,[58] proteases,[82] and chlorophylase.[83] When the intact plant leaf is first fragmented in the Waring blender, all the soluble components are brought into intimate contact with the particulate chloroplast fragments, exposing them to undesirable effects.

The effect of small amounts of rabbit sera was studied. Normal rabbit serum (NRS) and immune rabbit serum (IRS) diluted 1:100 in the 0.025 M Tris buffer (pH 6.97) had a negligible effect on the Hill reaction activity. However, when both NRS and IRS were diluted 5:100, activity was definitely greater. The chloroplast fragment preparations were refrigerated overnight at 4°C and tested for activity approximately

24 and sometimes 48 hr after preparation. The activity was slightly greater in preparations containing sera. Immune sera showed a slight advantage over normal sera. In each experiment, a control preparation was made using Tris buffer alone. Surprisingly, stored control preparations were also active.

These results suggest that enhancement might occur when any moderately high molecular weight solute is present when the Hill reaction components are released from the leaf structure. Some studies were performed in 0.4% gelatin-Tris buffer and 1% dextran-Tris buffer. Although the activity in the gelatin solution showed enhancement at 4 hr, there was a considerable decrease in activity at 24 hr. The dextran solution was higher in initial activity than the control and also showed improved activity after one day of storage.

Normal horse serum (NHS), when present in the buffer at a dilution of 5:100, enhanced the activity more than did NRS. Immunodiffusion analysis of IRS against the immunizing antigen from the chloroplast fragment supernatant solution indicated at least two precipitin arcs on micro-Ouchterlony plates. This was confirmed by immunoelectrophoresis of the supernatant solution. It is possible that the NHS used in these studies contains antibodies against plant antigens and that its enhancing activity is similar to that of IRS.

In an experiment using 5:100 IRS, the initial activity of $Q_{O_2}^{Chl}$ 450 increased to $Q_{O_2}^{Chl}$ 1215 one day after storage at 4°C. One of the problems of the immunochemical approach is the large quantity of IRS required for chloroplast fragment preparation. The minimum volume of solvent in the Warning blendor is approximately 50 ml. Hence, at least 3 ml of serum is used to prepare a colloidal buffer with enhancing activity. The data for several experiments are given in Table IX.

The results of sustained Hill reaction activity are encouraging. Since it appears that colloidal solutions of chloroplast fragments may withstand considerable manipulation, preparative electrophoresis will be applied to the more interesting, highly active centrifugal fractions

Table IX

EFFECT OF SERA AND COLLOIDS ADDED TO EXTRACTION MEDIUM ON HILL REACTION ACTIVITY

Item	Experiment 1			Experiment 2		Experiment 3				Experiment 4			Experiment 5		Experiment 6		
	Chl (mg/ml)	4 hr	24 hr	Chl (mg/ml)	4 hr	Chl (mg/ml)	4 hr	24 hr	48 hr	Chl (mg/ml)	4 hr	24 hr	Chl (mg/ml)	4 hr	Chl (mg/ml)	4 hr	24 hr
		Q O_2	Q O_2		Q O_2		Q O_2	Q O_2	Q O_2		Q O_2	Q O_2		Q O_2		Q O_2	Q O_2
Control	0.87	340	327	.87	200	.435	323	372	248	.206	262	262	.185	730	.228	237	237
1:100 NRS[a]	1.12	311	327	.581	248	----	---	---	---	----	---	---	----	---	----	---	---
5:100 NRS	----	---	---	.510	423	.255	424	494	407	----	---	---	.191	472	.223	323	382
1:100 ARS[b]	1.16	280	322	.535	314	----	---	---	---	----	---	---	----	---	----	---	---
5:100 ARS	----	---	---	.361	665	.181	696	796	497	----	---	---	.210	642	.200	450	1215
Gelatin, 0.4%	----	---	---	----	---	----	---	---	---	.357	412	353	----	---	----	---	---
Dextran, 1%	----	---	---	----	---	----	---	---	---	.197	290	412	----	---	----	---	---
5:100 NHS[c]	----	---	---	----	---	----	---	---	---	.201	413	538	.187	193	.214	449	505

[a] Normal rabbit serum

[b] Antibody rabbit serum

[c] Normal horse serum

such as CF_{20-50}. It should be possible to perform the electrophoresis with colloidal enhancing agents in the electrolyte.

The studies of various methods and substances for solubilization and protection of the Hill reaction system were not greatly rewarding. In general, the following conclusions were drawn:

1. Freezing proved a much more satisfactory method of storing active chloroplast fragments than lyophilization.

2. When used for solubilization, digitonin lowered Hill reaction activity immediately, and the fragments appeared to retain this lower level of activity when stored frozen.

3. A 10% solution of DMSO was found to have no inhibitory action on chloroplast activity and might be useful in future studies.

4. When 1% caffeine was included in the extracting medium, Hill reaction activity diminished.

5. With the exception of dextran, colloids such as rabbit and horse serum and gelatin produced a protective action, especially when the chloroplast preparation was stored at 4°C for a few days.

B. Electrophoretic Fractionation

One of the simplest methods of separating chloroplast preparations into fractions is by sedimentation, which can be performed rapidly enough to permit the study of Hill reaction activity in the fractions. Other biophysical procedures, however, are so time consuming that most of the activity is lost during fractionation.

We have gained considerable experience in using high voltage preparative electrophoresis on the carrier-free instrument, the Elphor FF, designed by Hannig. In contrast to curtain electrophoresis at 30 watts, chloroplast preparations can be studied easily on a preparative scale and under high power conditions of about 300 watts. Table X is a summary of operating conditions and the resulting spread of pigmented chloroplast materials into various fractions of the receiver assembly.

Table X

OPERATING CONDITIONS FOR PREPARATIVE ELECTROPHORESIS OF CHLOROPLAST PREPARATIONS

Expt. No.	Ma.	Volts	Dosing		Inlet Port	Buffer		Residence Time in Field (min)	Temp (°C)	Over-all Spread Between Fractions	TRIS Concentration (Molarity)
			Dial Setting	Rate (ml/hr)		Dial Setting	Rate (ml/hr)				
1	150	1950	1	3.0	38-39	2.5	153	41.7	4	19-29	0.05
2	150	1950	1	3.0	38-39	2.5	153	41.7	4	15-27	0.05
3	150	2000	2.0	4.8	38-39	2.5	153	41.7	4	16-35	0.05
4	150	1980	2.0	4.8	34-35	2.5	153	41.7	4	16-38	0.05
5	140	1850	4.0	8.6	34-35	2.5	153	41.7	4	15-37	0.025
6	140	1850	3.0	6.7	34-35	7.5	363	17.6	4	27-33	0.025
7	140	1850	3.0	6.7	34-35	2.5	153	41.7	4	19-33	0.025
8	0	0	3.0	6.7	34-35	2.5	153	41.7	4	33-37	0.025
9	140	1800	5.0	10.5	34-35	2.5	152	41.7	4	19-30	0.025
10	140	1850	4.0	8.6	34-35	2.5	153	41.7	4	17-29	0.025
11	70	1080	4.0	8.6	34-35	2.5	153	41.7	4	25-33	0.025
12	100	1450	4.0	8.6	34-35	2.5	153	41.7	4	23-34	0.025
13	200	2000	5.0	10.5	34-35	7.5	363	17.6	6	24-32	0.025
14	190	1940	5.0	10.5	34-35	7.5	363	17.6	6	23-33	0.025
15	170	1980	5.0	10.5	34-35	2.5	153	41.7	4	12-29	0.025

Whole chloroplasts (P_1) agglutinated spontaneously in the separation chamber during electrophoresis. Since this phenomenon was rarely observed with chloroplast fragments (P_2), preparations of them were studied in detail.

In general, most of the proteins in the mixtures migrated toward the anode in the pH 7.5 buffer. The suspensions gave green patterns that broadened as much as 23 cm from their inlet points (Experiments 4 and 5). During the residence period in the chamber, the suspensions formed characteristic streaked patterns. Effluent was uniformly dispersed green suspension that gave typical fluorescence when irradiated with 365 mμ light.

With the exception of Experiments 2, 3, and 4, all of the experiments were performed with chloroplast fragments (P_2). Digitonin fraction PD_2 was studied in Experiment 2, and the smaller digitonin fragments PD_3 were used in Experiment 3. The corresponding supernatant solution SD_3 was also studied; it gave the very broad pattern under the conditions of Experiment 4.

Experiments 6 and 7 show the effect of varied buffer flow rate. At the high flow rate, the residence time in the electric field is shorter. Consequently, there is less resolution (spreading), but the effluent contains a higher concentration of chloroplast fragments. Experiments 7 and 8 can be compared as a measure of the effectiveness of electrophoresis. Experiment 8 (no power) showed a maximum spread of five fractions collected in a direct line below the inlet port, whereas Experiment 7 (38 volts/cm for 42 minutes) produced a broad pattern of 15 fractions skewed far enough toward the anode to be clear of any components with negligible mobility, had they existed. In Experiment 9, the increased rate of sample dosing broadened the pattern slightly.

An old sample of chloroplasts extracted with Tris and stored at 4°C for 11 days was studied in Experiment 10-12. In contrast, no satisfactory separation was obtained with chloroplast fractions prepared with

the sucrose-phosphate buffer and stored at 4°C for 14 days before electrophoresis in the Elphor FF.

At a buffer flow rate of 153 ml/hr/48 tubes, the effluent is collected at 3.2 ml/hr/tube. A typical sample dosing rate of 6.7 ml/hr produces fractions containing chloroplast material that is four to eight times as dilute as the dosing solution. Consequently, some method of concentrating the effluent was comtemplated. However, it will be shown that concentration was not necessary.

Based on the preliminary studies of fresh and aged chloroplast preparations in the Elphor FF, the ultimate experiment was performed. It combined the typical preparation of chloroplast fragments, followed by preparative electrophoresis in the Elphor FF, and terminating in the manometric determination of Hill reaction activity in the electrophoretically separated effluent fractions. We would like to emphasize that it was crucial to carefully place a plastic bag containing ice over the plastic tubing from the dosing pump to the separating chamber. The pigment was spread over a range of 7 cm in the 48 fractions, from fraction 27 through 34. The green effluent from the Elphor FF subsequently fluoresced characteristically red when irradiated with light of wave length 365 mμ. The concentration of total chlorophyll, of chlorophyll a and b, and the Hill reaction activity in selected Elphor fractions are summarized in Table XI.

A second experiment involving an Elphor separation and Hill reaction determination was performed on an independent preparation of chloroplast fragments. A slightly higher electrolyte, pH 7.49, was used. The conditions in the Elphor FF separation were essentially the same, with the exception that the volts were 2100; consequently, the chloroplast fragments were subjected to 42 v/cm for about 18 min at 336 watts. Data for the second experiment are shown in Table XI.

The concentration of chlorophyll (a + b) in each of the effluent fractions from the Elphor was approximately four to five times as dilute as the concentration in the dosing solution (usually 1.5 mg

Table XI

CHLOROPHYLL CONCENTRATIONS AND HILL REACTION ACTIVITIES OF ELECTROPHORETICALLY SEPARATED CHLOROPLAST FRAGMENTS

Fraction	Chlorophyll (mg/ml)				$Q_{O_2}^{Chl}$
	Total		Chl a	Chl b	
	Approx.	Precise			
pH 7.05					
Control	1.30	1.40	1.05	0.35	406
30	0.280	0.287	0.211	0.076	729
31	0.343	0.334	0.252	0.082	525
32	0.278	0.270	0.202	0.068	712
pH 7.49					
Control	1.60	1.39	0.910	0.480	327
27	0.330	0.291	0.183	0.108	424
28	0.440	0.389	0.245	0.144	567
29	0.410	0.368	0.233	0.135	382
30	0.294	0.263	0.153	0.110	433
31	0.267	0.238	0.148	0.090	472

chlorophyll/ml). Even at this dilute concentration, it was possible to use the effluent directly for the manometric measurement of Hill reaction activity without a concentration step. All of the fractions showed Hill reaction activity. The middle fraction of pigmented effluent had a lower Hill reaction activity than fractions adjacent to it. This effect was noticeable in both experiments.

Some separation of crude components occurred in our experiments. For example, electrophoretically diverted chloroplast fragments exhibited higher specific activity than did unseparated fragments. The reproducibility of lower activity of fractions from the middle region in the electrophoretic pattern is interpreted to indicate a chemically fragile fraction or one lacking components in optimal proportions. On the other hand, it should be noted that chloroplast fragments bearing chlorophyll a were not resolved from those containing chlorophyll b.

There are several useful features about the Elphor separation. Whole chloroplasts, chloroplast fragments, and even soluble components can be studied conveniently, at low temperatures, under neutral pH, and on a preparative scale. Moderately concentrated suspensions may be studied, i.e., mixtures of chloroplast fragments were applied directly into the Elphor. Some dilution was incurred by the separation, but it was not necessary to apply a concentration step, such as ultrafiltration or concentration-dialysis before the effluent could be examined for Hill reaction activity. The experiment can be performed relatively rapidly. Although the chloroplast fragments were in the electrophoretic field for only 18 min, longer residence periods are possible. At the upper limit (about 3 hr), the extreme conditions may prove useless against such fragile, biologically active materials with relatively short half-lives. In the Elphor, it is possible to use a variety of buffer systems, selecting those that provide a minimum of anionic uncoupling of the type described by Good.[84] Buffers containing other potential stabilizing agents, such as carbowaxes, dextrans, and polyvinylpyrrolidone are compatible with the Elphor. Fredericks and Jagendorf[67] recently described an extractable protein required in

the Hill reaction in Anacystis nidulans. Carbowax 4000 or dextran and calcium ions not only preserved the Hill reaction activity but gave marked stimulation. From our experience, these factors do not appear to play a role in the Hill reaction system of spinach chloroplast fragments because of the notable absence of tannins. However, the presence of the cofactors and protective agents could be included during Elphor separations.

Until recently, the only preparative electrophoretic method involved a solid support, such as a paper curtain.[85] Unfortunately, the chloroplast pigment-protein complex has a marked affinity for cellulose. All of our previous attempts to study the mobility of chloroplast fragments by paper strip or paper curtain electrophoresis have been unsuccessful because the sample remains at the point of application.

Using the Elphor FF for the separation of chloroplast fragments is not without problems. The critical sensitivity of Hill reaction catalysts to light demands that experiments be performed in dim green light. Another problem is encountered in the method of collecting effluent from the Elphor FF: From 48 small collecting wells adjacent to the chamber, effluent is periodically aspirated into larger collector tubes in an air-cooled receiver. A nitrogen, rather than oxygen, atmosphere would favor fractions with higher Hill reaction activity.

The literature contains some references to electrophoresis of pigment-protein complexes. Svensson and Brattsten[86] and later Brattsten[87] described the continuous electrophoretic separation of the algal billi-chromoproteins phycoerythrin and phycocyanin. Two chromoglobulins containing phosphate groups were included among six or seven components

electrophoretically resolved from chloroplast proteins by Sisakyan and Melik-Sarkisyan.[88] Eversole and Wolken[89] described chloroplastin, a digitonin-extracted chloroplast fragment preparation, as electrophoretically homogeneous; however, no details were given. A description of experimental conditions and results was omitted by Heber,[90] who claimed to have achieved Hill reaction activity of small chloroplast fragments separated by curtain electrophoresis.

Gross, Becker, and Shefner[91] reasoned that it might be possible to isolate, from a centrifugal fraction of chloroplast fragments prepared by osmotic shock, a chemically and structurally pure fraction that might be responsible for the high Hill reaction activity of the parent mixture and that would exhibit an even higher reaction rate. The electrophoretic method of Kolin[92-94] was used in attempts at further fractionation. However, the volume of the fractions withdrawn from the Kolin cell was so small that there was insufficient material for assay of both the chlorophyll content and Hill reaction activity. While the Kolin method may ultimately prove rapid, convenient, and sensitive for qualitative analysis of any biophysical separation, it is incomparable with the preparative scale afforded by the Elphor FF.

Recently, the preparative electrophoretic separations of plant particulates by Hannig, Klofat, and Endres[95] came to our attention. Their spinach homogenate gave a much narrower separation pattern in the Elphor than we have reported even though their voltage and power were higher. We tentatively conclude that the pH was low enough to restrict the mobility of the chloroplast fragments. It was unfortunate that an attempt was not made to demonstrate Hill reaction activity.

A general problem of determining Hill reaction activity is the sensitivity of the detecting system. Standard Warburg manometry is an extravagant method to study the catalytic activity of photosynthetic systems; large amounts of the bio-catalysis (chloroplast fragments containing chlorophylls, etc.) are required to demonstrate activity by oxygen evolution. Among the alternative micro methods, the platinum

electrode oxygen detector of Blinks and coworkers,[96,97] as employed by Fork,[98] offers many advantages to improve sensitivity and oxygen specificty while conserving the bio-catalyst. Consequently, we changed our Hill reaction analysis to this method of detection. According to Spikes,[99] Hill reaction activity is inversely related to the initial concentration of oxidant. Results reported by Arnon and Whatley[77] strongly support the suggestion that smaller quantities of reactants and catalysts should facilitate higher Hill reaction rates.

This method of sensing the evolution of oxygen was used in a series of experiments in which the Hill reaction activity was determined as a function of the volume of chloroplast fragment suspension added as the biocatalyst. The results for a series of preparations are summarized in Table XII.

Table XII

EFFECT OF CHLOROPHYLL CONCENTRATION ON HILL REACTION ACTIVITY MEASURED WITH THE POLAROGRAPHIC OXYGEN-SENSING ELECTRODE[a]

Experiment Number	$Q_{O_2}^{Chl}$			
	Volume of Chloroplast Suspension (ml)[b]			
	1.00	0.50	0.25	0.125
1	339	528	---	---
2	---	323	456	---
3	---	150	286	---
4	536	562	715	---
5	378	510	641	782
6	268	---	524	---

[a] Influenced by the quantity of biocatalyst added to the reaction mixture.

[b] All experiments were conducted with a total volume of solution of 3.00 ml in the 15-ml Warburg flasks.

In general, the slope of the curve of Hill reaction activity is a function of chlorophyll concentration in the system and indicates increasing activity as the concentration of biocatalyst approaches infinite dilution. Unfortunately, the data are too incomplete to permit useful evaluation. In future experiments, we plan to study the variation of activity at a fixed level of chlorophyll concentration, e.g., 0.50 or 0.25 mg chlorophyll per ml and to study the shape of the curve in greater detail at lower concentrations of chlorophyll.

One explanation frequently offered is that some chloroplast fragments screen others from receiving adequate illumination. Consequently, the Hill reaction activity is proportional to the efficiency of exposure of the biocatalyst. This would be improved at higher dilutions at a sacrifice of precision. However, there should be a concentration below which the activity is a linear function of the biocatalyst concentration, i.e., free of screening interference.

Another possiblity is suggested by the Schütz rule--it is necessary to quadruple the quantity of the enzyme in order to double the rate of reaction.[100] Assuming that the concerted enzymatic activity in the Hill reaction is directly related to the quantity of chlorophyll, the results very closely resemble those typical of the Schütz phenomenon. Further studies of the reproducibility of results will help in the adoption of this interpretation.

The studies with the polarographic electrode were performed with a single flask and its electrode. It should be possible to monitor several flasks simultaneously through a multipoint recorder.

Since Gross and coworkers[73-76] had characterized a specific centrifugal fraction of sonically disrupted chloroplast as having the greatest Hill reaction activity, we repeated their methods of sonication and centrifugation. The sonically disrupted chloroplast fragments were readily separated into three fractions: P_{0-20}, P_{20-50}, and S_{50}. The spectra of the latter two fractions were obtained in aqueous Tris buffer. There is a distinct absorption difference between them only

in the ultraviolet region but not in the visible region. These spectral differences are best seen by comparison of the overlays in the envelope at the back of the report. The original spectra were adjusted proportionately to a relative absorbance of 1.000 at 678 mμ, the maximum of the chlorophyll spectrum in this aqueous medium.

In several experiments, the P_{20-50} fraction was separated electrophoretically in the Elphor FF. The separation was similar to that of the heterogeneous fraction called chloroplast preparation. An important difference was the spread of maximum chlorophyll concentration over a 3 cm distance in comparison with only 2 cm in the heterogeneous pattern. Preliminary results indicated that the P_{20-50} fraction gave an Elphor separation without the minimum of Hill reaction activity so noticeable in the heterogeneous pattern. Additional studies are required to clarify this result. In accordance with the conclusions of Park and Biggins,[101] we tentatively conclude that this refined centrifugal fraction may be devoid of some permeability membrane that restricts Hill reaction activity of the components in the middle of the electrophoretic pattern of the heterogeneous chloroplast preparation.

Another effect was observed and is attributed to competition of proteins for the energy in the electrophoretic field. If previously separated fractions, e.g., Fraction 21 and 22, are rerun through the Elphor FF under the same experimental conditions as they were obtained, the pattern is effectively broadened toward the anode, although the mobility of components on the cathodic edge of the pattern remained the same as in the previous separation.

An additional set of overlays for the spectra of the electrophoretic fractions of a P_{20-50} fraction are included in the envelope at the back of the report. They may be compared with each other and against the master spectrum of Fig. 2, appended to the report.

In conclusion, we have readily established that chloroplast fragments, produced by mechanical and sonic disruption, retain Hill reaction activity after preparative electrophoresis. As with heterogeneous

chloroplast fragments, a minimum of Hill activity is associated with chlorophyll-rich components in the middle of the electrophoretic separation pattern; this is probably not the case in the highly active P_{20-50} fraction. The size of the chloroplast particle has little, if any, effect on electrophoretic mobility. Spectral characteristics are almost constant and are independent of electrophoretic mobility.

A fruitful area for research, however, is the nature of the proteins associated with the pigments and lipids of the photoactive particles. The proteins can be studied in terms of structure and composition after the pigments and lipids have been removed by extraction with organic solvents. Park and Biggins,[101] have described the quantasome with a molecular weight of 2×10^6. One half of it was lipid, including a very complex inventory of components. The other half of the quantasome was protein. It seems highly likely that the protein will be an equally complex array of protein components. Electrophoresis should assist in fractionation studies.

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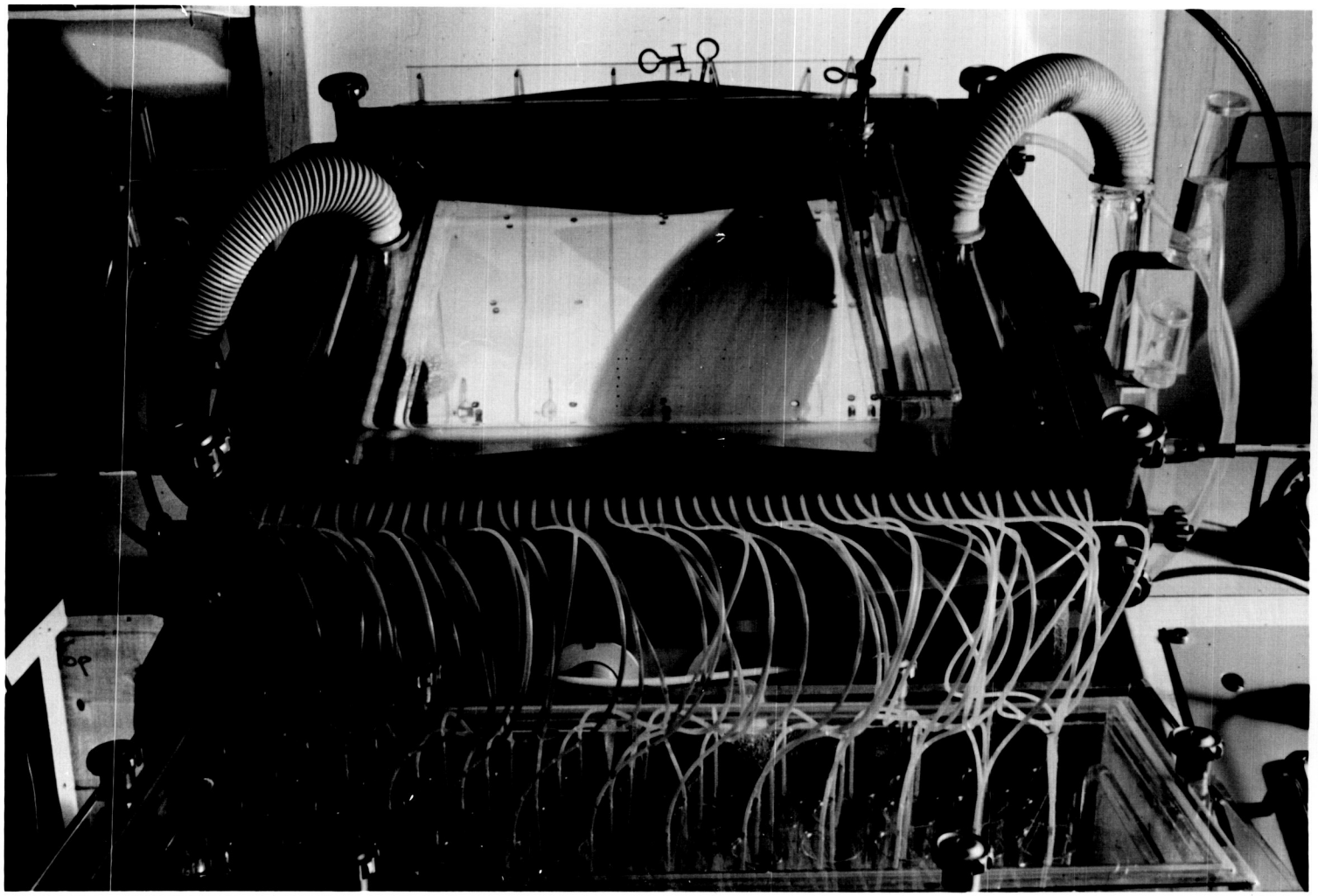

FIG. 1 ELPHOR FF ELECTROPHORETIC SEPARATION OF CHLOROPLAST PREPARATION

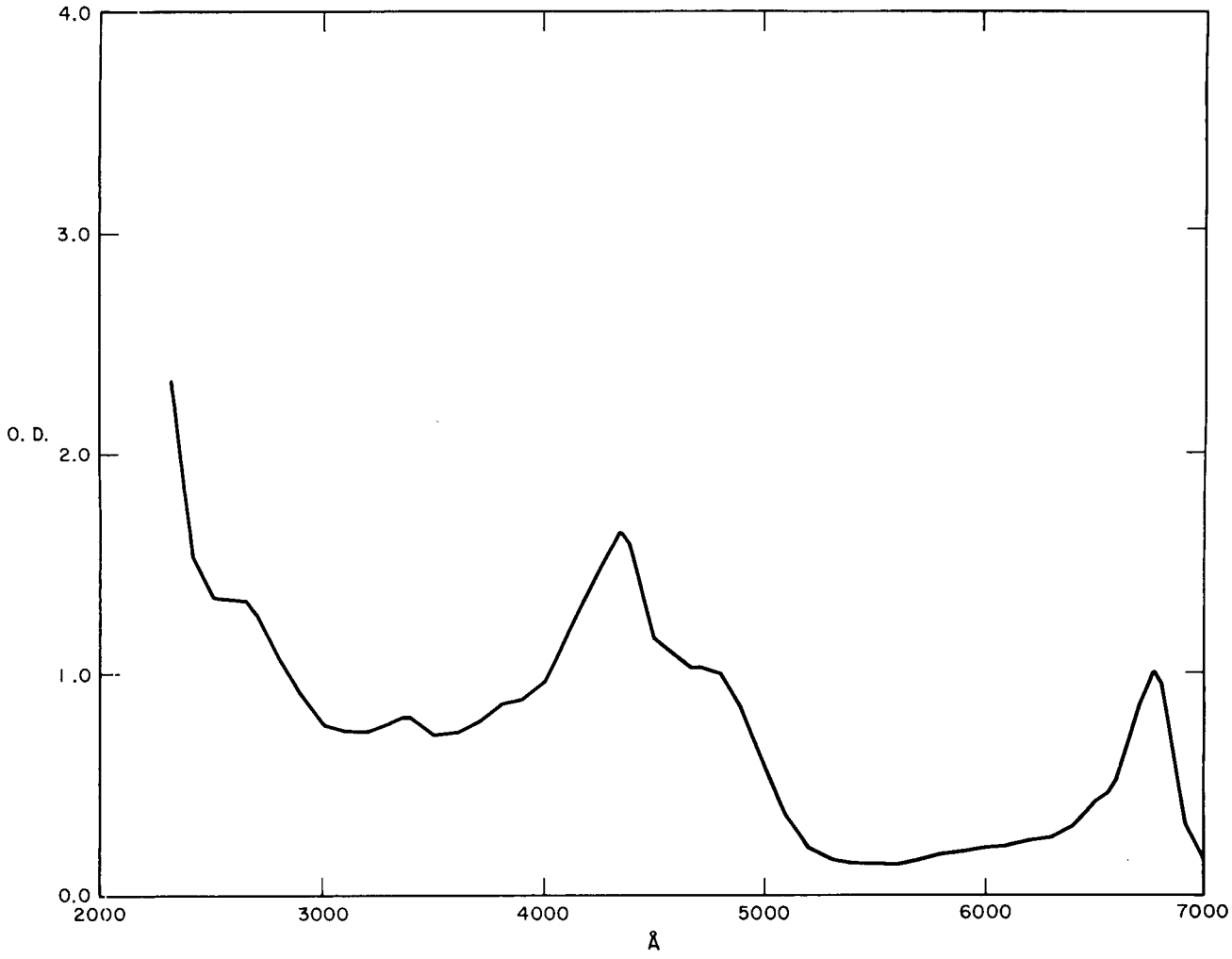

FIG. 2 CHLOROPLAST PREPARATION MASTER SPECTRUM