STABILITY OF ISOLATED CHLOROPLAST PREPARATIONS AND ITS EFFECT ON HILL REACTION MEASUREMENTS[1,2]

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Since the time of the discovery and the first characterization of the Hill reaction (13, 14) there have been many subsequent studies of this process. As might have been expected, the results obtained in these different studies are rather contradictory, so much so that it is not clear what the characteristics of the Hill reaction are. This point is well illustrated in the complete review of the older Hill reaction work found in Rabinowitch (30).

From a preliminary consideration of this problem (26) it seemed possible that the contradictions in the literature were due to the use of intact chloroplasts by some workers and fragmented chloroplasts by others. The results obtained in this study have made it clear, however, that the observed properties of a chloroplast preparation are strongly influenced by a number of factors such as the stability of the material, the species plant from which the chloroplasts were isolated and its physiological condition, and the time needed to make a measurement, to name but a few. Additional complications have been introduced by the recent finding that phosphorylation can be an integral part of the Hill reaction (2). The purpose of this paper is to fit together the pieces of this puzzle, insofar as possible, and show that one description of the Hill reaction will suffice to explain the results obtained in different studies.

MATERIALS AND METHODS

The higher plants from which chloroplasts were isolated were field grown. The leaves were picked the day they were used and were kept in a water saturated atmosphere until they were ground. Elodea sp. and Stratiotes sp. were grown in an aquarium with a layer of sand-covered soil on the bottom. The tank was illuminated by fluorescent lights with an intensity of about 100 ft-c. The lights were controlled by a timer so that a cycle of 16 hours light, 8 hours dark was maintained. *Chlorella pyrenoidosa* and *Chlamydomonas reinhardi* were grown in glass culture flasks under an atmosphere of 5 % CO_2 in air and kept at a temperature of 15 to 18° C by a water bath. Continuous illumination of about 200 ft-c was provided by fluorescent lights. In several experiments, high intensity white light (about 1500 ft-c) was provided by a 200-watt incandescent bulb, the light from which was filtered and focused by a 2-liter water-filled florence flask. The culture medium for Chlorella was that used in Emerson's laboratory (26) and the medium for Chlamydomonas was that of Granick and Sager (11) except that 20 μM/l $FeSO_4$ was used instead of ferric citrate and the micronutrients were modified A 5 and B 9. (26).

For the preparation of higher plant chloroplasts, washed turgid leaves were cut into pieces about 1 cm square. These were then ground with a mortar and pestle in buffer containing the following: 0.03 M phosphate usually at pH 7.0, 0.33 M glucose and 0.01 M KCl, unless otherwise specified. The suspension was filtered through Pyrex glass wool, centrifuged for 90 seconds at 100 G to remove whole cells and other debris, then decanted and centrifuged for 5 minutes at 900 G to bring down whole chloroplasts. This fraction was resuspended in the same buffer and centrifuged again before suspending in the final medium. Fragmented chloroplasts were removed from the whole chloroplast supernate by centrifugation at 18,000 G for 30 minutes. The supernate was decanted and discarded, and the tube rinsed with fresh buffer before resuspending the pellet. The chloroplast fragments so prepared were used without further washing.

Algal chloroplast fragments were prepared by mixing washed packed cells with kieselguhr and grinding with a mortar and pestle. The mixture was slowly resuspended in buffer and centrifuged for 5 minutes at 1000 G to remove the abrasive and whole cells. The supernate was then treated as described above for higher plant chloroplast fragments. All chloroplast preparations were carried out at temperatures between 0 and 4° C.

The photochemical activity of the chloroplasts was determined by measuring the rate of reduction of the dye 2,6, dichlorophenol indophenol, or of potassium ferricyanide. The ferricyanide was prepared immediately before use in distilled water. The concentration was measured optically assuming the molar extinction coefficient ($\log_{10}$) to be 980 at 420 mμ. The dye was purified according to the following method suggested by Dr. R. Hill: A small amount of dye, dissolved in 0.03 M phosphate buffer pH 7.1, was extracted with ether in a separatory funnel. The ether containing the acid form of the dye was filtered through double filter paper and then extracted with 0.01 M Na_2CO_3 solution until all the dye was in the aqueous phase. The dye was then salted out with NaCl, collected by filtration and air dried. The molar extinction coefficient ($\log_{10}$) of this preparation was found to be 22,400 at 610 mμ according to the method

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of Rosin (31) and was 95 % pure assuming it to be a dihydrate (N. Savage and T. Punnett, unpublished data). Because the absorption of the dye changes with pH (pK = 5.7) all changes in optical density were multiplied by the following empirically determined factors: 1.23 at pH 6.5, 1.09 at pH 6.9, 1.05 at pH 7.2, 1.00 at pH 8.0 and higher.

The photometer had 2 light paths at right angles to each other (fig 1). The light from the 250-watt mercury lamp was stabilized by a power supply (fig 2) which in turn was controlled by a photocell monitoring a portion of the output of the mercury lamp. The mercury light passed through a heat filter, and through Wratten filters which isolated the 577/579 mμ line for dye experiments or the 405 mμ line for ferricyanide experiments. The output from the measuring beam photocell was amplified by a battery operated D.C. amplifier (fig 3) and fed to a galvanometer. The light from the tungsten lamp passed through a heat filter and a red filter with a cut-off at 620 mμ. This light provided the energy for the Hill reaction and caused almost no response on the violet sensitive photocell. Galvanometer readings were taken at 15 or 20 second intervals and the final reading determined after complete reduction of the dye by ascorbic acid. The rate of change of optical density was calculated from these figures and was converted to $Q_{O_2}^{ch}$. The chlorophyll concentration in the reaction vessel, determined by Arnon's modification (3) of MacKinney's method (24), was usually between 1.0 and 8.0 μg of chlorophylls a plus b per ml.

Results

One of the first phenomena noticed in this study was that intact and fragmented chloroplasts were stable under very different conditions (27). The stability of a preparation was determined from a plot of log $Q_{O_2}^{ch}$ vs time and expressed as half-life of the photochemical activity in hours. The error in the determination of any one half-life was about 5 to 10 %, though there was often a variation of 50 to 80 % from day to day.

From the results obtained with pokeweed (*Phytolacca americana*, L.) (table I) it can be seen that whole chloroplasts in glucose (row 2 of table I) were at least 10 times more stable than the control (row 4). Fragmented chloroplasts in glucose (row 6) were only twice as stable as the control (row 8), a difference not significantly greater than error. Both fragmented and intact chloroplasts were 5 to 10 times more stable in chloride medium than in buffer alone. Furthermore, when intact chloroplasts prepared in glucose buffer were put into low osmotic pressure buffer and then returned to the glucose buffer, they no longer could be stabilized by glucose. The simplest interpretation of these results is that the high osmotic pressure glucose medium preserves the integrity of the chloroplast membrane. Once the membrane is irreversibly damaged, for example by osmotic shock, the photochemical system is inactivated rapidly unless chloride is present.

Table I

Half-life of Hill Reaction Activity (hrs) of Pokeweed Chloroplasts

Storage medium supplement	pH of storage medium 6.5	6.8	7.1	7.4	7.7	8.0	8.2
			intact chloroplasts				
1 Gluc + Cl	70	52	37	31	19	4.0	2.8
2 Glucose	73	61	200	53	5.7	2.1	1.3
3 Chloride	...	28	27	...	...	<0.6	...
4 None	...	5.5	5.5	...	...	<0.4	...
			fragmented chloroplasts				
5 Gluc + Cl	...	20	16	...	...	1.0	...
6 Glucose	...	5.5	...	...	...	<0.4	...
7 Chloride	11	16	20	23	5.0	1.4	<0.7
8 None	2.5	2.7	1.8	1.7	<0.6	<0.6	<0.6

Experiments done in 2 successive days therefore 50 % to 80 % variation between rows. Activity measured at pH 8.0. Reaction conditions as specified in Materials and Methods.

When the stability of chloroplasts from other species was determined, the results were often different. For example, chloroplasts from Good King Henry (*Chenopodium Bonus-Henricus*, L.) did not show an appreciable glucose effect (table II) although there was a pronounced chloride effect. This lack of an "intact chloroplast" effect was rather common since it was also found with pea (*Pisum sativum*, L.), and chard (*Beta vulgaris*, L.) (table III). This is consistent with the membrane hypothesis mentioned above because in these preparations, there was a great deal of starch, and starch grains are known to tear through the chloroplast membrane during centrifugation (21).

With chickweed (*Stellaria media* Cyrill), the chloroplasts were glucose stabilized only in alkaline media (pH 7.7 to 8.2) and the maximum half-life (6.0 hrs) was found at pH 7.2. In agreement with the earlier results of Hill ((15), and personal communication) it was also found that the pH of the grinding medium had to be high (ca. 7.7) in order to obtain active chloroplasts, although the chloroplasts were most stable at pH 7.2 after isolation. Recently Jagendorf and Evans (20) have shown that the beneficial effect of an alkaline grinding medium is rather general, and could be demonstrated with 5 of the 14 species they examined.

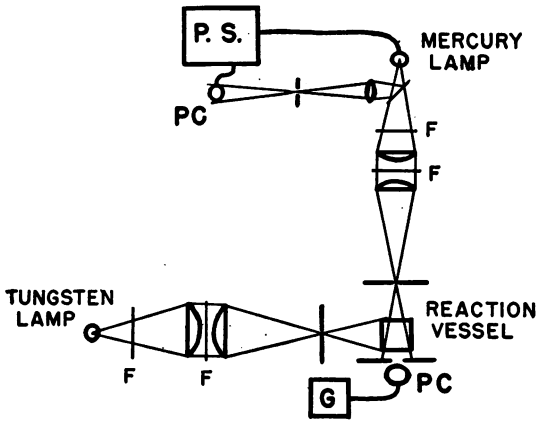
In addition to the dependence of stability on species, both the stability and the activity of chloroplasts from field grown plants were found to change during the

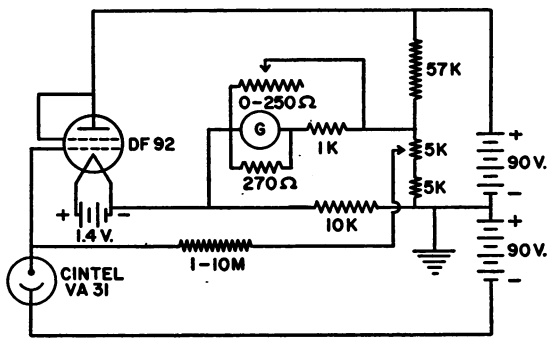
Table II

Half-life of Hill Reaction Activity (hrs) of Intact Good King Henry Chloroplasts

Storage medium supplement	pH of storage medium 7.0	7.7	8.1	8.3
1 Gluc + Cl	20	20	6.0	3.5
2 Glucose	12	6.0	3.0	...
3 Chloride	40	40	5.0	<3.0
4 None	6.5	2.5	<2.0	...

Experiments done in 1 day. Error between rows 5 % to 10 %. Activity measured at pH 7.8. Reaction conditions as specified in Materials and Methods.





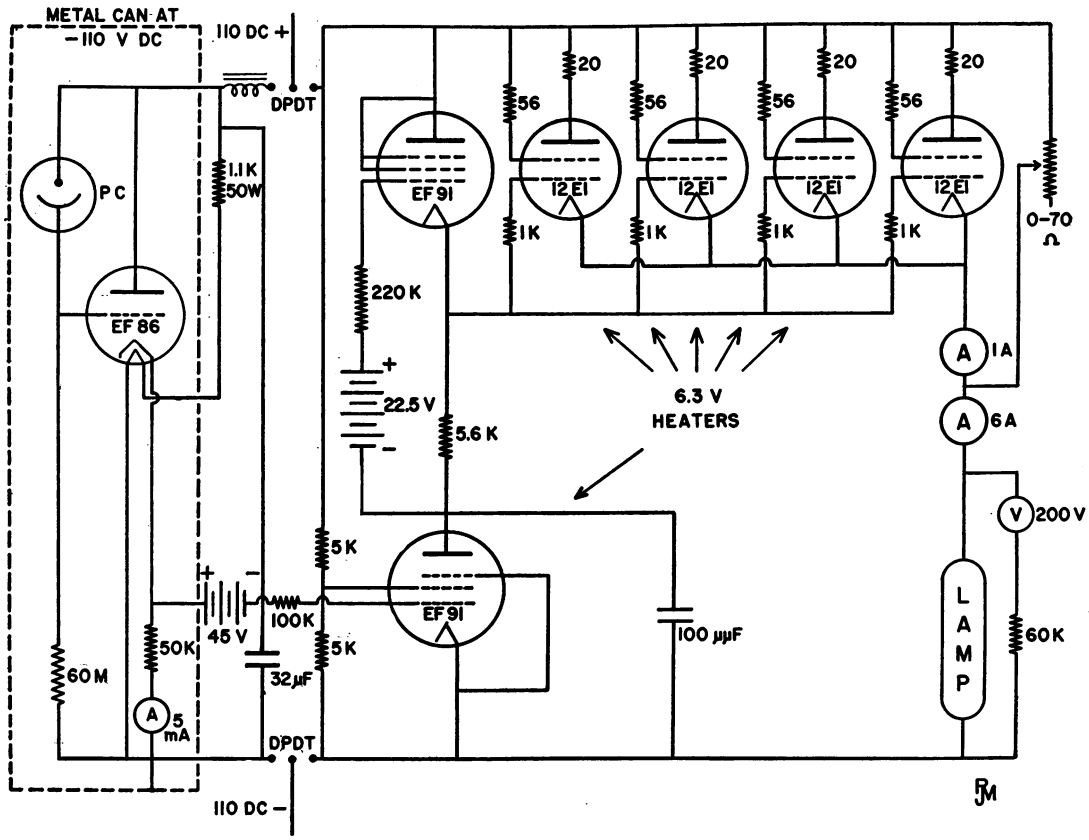


FIG. 1 (*upper left*). Diagrammatic sketch of double beam photometer.

FIG. 2 (*bottom*). Circuit diagram of feedback-stabilized D.C. power supply for mercury lamp.

FIG. 3 (*upper right*). Circuit diagram of D.C. amplifier and galvanometer for measuring beam photocell.

TABLE III

HALF-LIFE OF HILL REACTION ACTIVITY (HRS) OF INTACT CHARD CHLOROPLASTS

STORAGE MEDIUM SUPPLEMENT	pH OF STORAGE MEDIUM 6.8	7.3	7.7	8.1
1 Gluc + Cl	7.0	10	3.6	2.3
2 Glucose	5.0	6.0	2.1	1.7
3 Chloride	10	15	5.0	2.3
4 None	3.5	3.5	1.1	<0.8

Experiments done in 1 day. Variation between rows 5 % to 10%. Activity measured at pH 8.25. Reaction conditions as specified in Materials and Methods.

growing season and also seemed to depend on the growing conditions. This may explain some of the variations in activity reported by Clendenning et al (8, 9) as well as the diurnal variation observed by Hill and Scarisbrick with intact chickweed chloroplasts (16) which could not be confirmed when the experiments were repeated with fragmented pea chloroplasts ((15), and personal communication). Whether the variation in activity due to preillumination (23, 25) and the effect of red light on Hill reaction activity reported by Appleman et al (1) are also due to differences in stability remains to be seen.

The hypothesis that chloride and glucose are effective because they stabilize the material (see (34)) rather than "restore the activity" or "stimulate the Hill reaction" (12, 35) was tested in another way. Pokeweed chloroplasts, whose activity was found to be $Q_{O_2}^{ch} = 1600$ in chloride buffer, were tested for Hill reaction activity in buffer alone. After 1 minute, the reaction was stopped and chloride added to a concentration of 0.01 M. The cuvette was immediately replaced in the photometer and the reaction was resumed. The activity was 750 before the addition of chloride, and 700 afterwards. Thus there was no sign of restoring the rate to the original level, but rather a slight loss of activity possibly due to the additional mechanical agitation. Similar experiments done with glucose gave the same results. This explanation of the chloride effect differs from that of Arnon (4) only in that thermal inactivation is involved rather than photo-inactivation.

In connection with these stability experiments, it should be pointed out that the activity of the preparations was measured at "the optimum pH," usually between pH 7.7 and 8.1. These experiments were therefore comparable to those of Hill and Scarisbrick (16) and quite different from those of Warburg (35) and of Arnon (5) done at pH 6.5 to 6.8 where the photochemical activity is much lower and the stability considerably greater. This inverse relation between stability and activity was shown by the results of an experiment in which the pH curve for the Hill reaction of chard chloroplasts was measured at successive intervals (28). The values for the half-life of the Hill reaction activity as a function of pH (table IV) have been calculated from these data (fig 4) as well as the activity extrapolated to time zero.

As can be seen from figure 4, there was no optimum for this exceptionally stable preparation, and indeed there probably is no true optimum for dye reduction. The optima found in different experiments merely reflect the amount of inactivation that has occurred befor the measurements are completed. This suggestion is consistent with the finding that the pH optima for the Hill reaction of chickweed, Good King Henry, pokeweed and pea chloroplasts varied from 7.7 to 8.5 from month to month, depending on the activity and the stability of the preparations. This variation in optimum did not depend on the use of fragmented vs intact chloroplasts, nor did it depend on the use of ferricyanide instead of the dye. For some reason not yet understood, we never observed the broad pH optimum at 7.1 recently found by Avron et al for very high rate ferricyanide reduction by "dilution activated" chloroplasts (7).

The greatest variation in pH optimum observed with any single species was found with chard. In early Spring the optimum was 6.9 and the activity was low ($Q_{O_2}^{ch}$ of 200 to 400), but by July the optimum rose to >8.7 and the $Q_{O_2}^{ch}$ was 2100. With chloroplasts isolated from *Chlorella pyrenoidosa* Chick, *Chlamydomonas reinhardi* Dang., 2 varieties of Elodea sp. Michx. and Stratiotes sp., the optimum was always low (ca. 6.9) as was the activity ($Q_{O_2}^{ch}$ 200 to 800). This may be due either to a species-dependent difference in the stability, or to the effect of growth under artificial light, or both. An attempt to increase the activity of the Chlorella chloroplasts by growing

TABLE IV

HALF-LIFE OF HILL REACTION ACTIVITY AS A FUNCTION OF pH*

pH	HALF-LIFE (HOURS)
6.5	130
6.6	90
6.9	120
7.2	175
7.5	95
7.8	85
7.95	48
8.1	27
8.25	23
8.4	13
8.55	9.5
8.7	7.4

* Fragmental chard chloroplasts stored in buffer containing 0.01 M KCl, 0.03 M phosphate buffer at pH 7.1.

TABLE V

EFFECT OF PREINCUBATION IN ALKALINE MEDIUM ON MANOMETRICALLY DETERMINED HILL REACTION RATES

pH OF TREATMENT		HILL REACTION RATES ($Q_{O_2}^{ch}$)	
PREINCUBATION	MEASUREMENT	CHARD	POKEWEED
6.8	6.8	735 ± 35	470 ± 25
8.1	8.1	0.0 ± 35	0.0 ± 25
6.8	8.1	200 ± 35	810 ± 25

the cells in high intensity light (ca. 1500 ft-c) proved to be unsuccessful.

The demonstration that the Hill reaction system is labile in alkaline media implies that a rapid measuring technique should be used in order to avoid interference due to inactivation. It further implies that when a slow technique such as manometry is used, it is likely that the results obtained are distorted. This may account for the fact that with one exception (17, 18), manometrically determined pH optima have been between 6.5 and 7.1. This suggestion was tested by measuring Hill reaction rates at pH 6.8 and 8.1 using the conventional manometric technique. In a 3rd vessel, the chloroplasts suspended in dilute buffer at pH 6.8 were placed in the side arm. They were tipped into the main compartment of the vessel containing buffer at pH 8.1 just before the vessels were illuminated. The rates obtained (tableV) showed that a 10-minute preincubation of the chloroplasts at an alkaline pH completely inactivated the material. On the other hand, if the chloroplasts were preincubated at pH 6.8, they retained some activity, and in the case of the more stable pokeweed showed the same pH response found photometrically.

Discussion

The results obtained in this study serve 2 purposes; they simplify greatly the interpretation of past Hill reaction studies, and they contribute to our understanding of the mechanism of the process.

One fact of primary importance for the analysis of data obtained in this and previous studies is that phosphorylation can be an integral part of the Hill reaction (2, 6, 7). As recently shown by Jagendorf and coworkers, the phosphorylation which accompanies ferricyanide reduction can be uncoupled by acid treatment (pH 6.0) or the addition of ammonium ion (19). Furthermore, dye reduction is almost independent of phosphorylation (7). For these reasons, the results obtained in this study did not depend on phosphorylation since either dye was used as the oxidant, or ferricyanide reduction was already uncoupled and proceeded at a high rate. This uncoupling was probably due to the low pH of the grinding buffer (22). For similar reasons, it appears that phosphorylation did not affect the results obtained in most other studies.

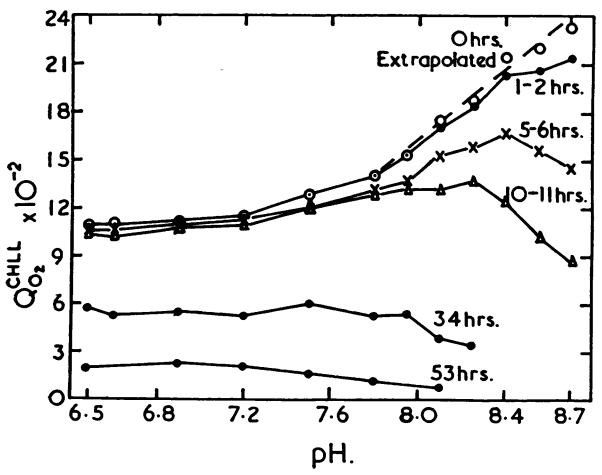


FIG. 4. Curves showing the change in the pH optimum of the Hill reaction accompanying inactivation. Curve for initial activity was determined by plotting log Q_{O2}^{ch} vs time and extrapolating to time zero.

In retrospect it becomes clear that the contradictions concerning the chloride effect, the glucose effect, the need for intact chloroplasts, the direct reduction of ferricyanide and the pH optima are more apparent than real. The chloride effect, which is due to the stabilization of fragmented chloroplasts was found in 5 different laboratories (4, 5, 12, 27, 29, 34, 35) but not by Hill et al (10, 15, 16). This is explained by the observation that the former workers used fragmented chloroplasts or "whole chloroplasts" damaged by treatment with dilute buffer, whereas Hill et al used intact chloroplasts in their early work. The reason for the lack of a chloride effect in their later study done with Good King Henry and pea chloroplast preparations (10) is not so clear, but may have been due to stabilization of the material by the added proteins. On the other hand, the glucose effect which is due to the stabilization of intact chloroplasts was found by Hill (14) and by Holt and French (17, 18) who omitted chloride from the medium thereby insuring rapid inactivation of any fragments present. Similarly Hill's original report that only intact chloroplasts were active stems from his use of chickweed chloroplasts which lose activity extremely rapidly if damaged or fragmented in the absence of chloride. The problem of the direct reduction of ferricyanide was solved by Jagendorf and Krogman's demonstration that treatment of chloroplasts with low pH buffer (pH 6.0) uncouples phosphorylation (22). It can be seen that in all cases of direct reduction of ferricyanide, the preparation medium was unbuffered or buffered at a low pH (5, 8, 9, 29, 32, 34, 35 and this study). In the work of Hill et al (15, 16) ferricyanide reduction was almost completely blocked by an intact but inoperative phosphorylation system. The reasons for the different pH optima that have been reported have already been discussed (28). Implicit in that discussion was the concept that high activity was always associated with a high pH optimum, as demonstrated by the change in the pH response accompanying inactivation. On this basis, all low pH optima (6.5 to 7.2) could be accounted for as inactivation artifacts due to preparatory techniques or measuring techniques, or both. However, this concept must now be modified because of the recent reports that under special circumstances very high rate ferricyanide reduction (7) and cyclic phosphorylation (33) have pH optima at 7.1 with a shoulder at 7.7.

When any attempt is made to reconstruct the mechanism of the Hill reaction, it becomes clear that the system is very complex. On the basis of the results obtained in this study, it is possible to distinguish between 2 (enzymatic?) steps. The one which is normally rate-limiting in active material, enzyme a,

has the pH response shown in the top curve of figure 4 suggesting a pK at 7.5, and is relatively stable. During storage, another enzyme, enzyme b, becomes rate-limiting. Enzyme b has a pH optimum at 6.9, is stabilized by chloride ion and is relatively labile. Furthermore, the pH curves of Avron et al (7) indicate that when the phosphorylation step is limiting the rate of ferricyanide reduction, the pH curve resembles neither of the two mentioned above. This points to the existence of a third step which can be rate-limiting.

Unfortunately, this evidence deals only with the limiting steps and gives no information regarding another important problem, the question of the number of chemically different reducing sites in the chloroplast. Considering the different possibilities, it is clear that if there is only 1 reducing site, then oxidants such as ferric oxalate, the methemoglobin reducing factor and dichlorophenol-indophenol must also be uncoupling agents. On the other hand, if there are 2 or more sites, it is necessary that these oxidants react with some reducing site prior to the phosphorylation step. Furthermore, both sites must occur after the above-mentioned rate-limiting steps because the pH curves do not vary depending on the oxidant.

In conclusion, it is hoped that the resolution of many of the contradictions concerning the Hill reaction simplifies the problem of analyzing the mechanism and throws the remaining problems into sharper relief.

Summary

1. Glucose and chloride stabilize the Hill reaction system of intact and fragmented chloroplasts respectively, and do not "restore" or "stimulate" the photochemical reaction.

2. The relative stability of the Hill reaction system of intact and fragmented chloroplasts is strongly dependent on species, and probably on growth conditions.

3. The method of measuring Hill reaction activity must be rapid because at an alkaline pH where dye reduction is most rapid, the Hill reaction system is very labile.

4. The results obtained in this study are consistent with and help to resolve most of the apparent contradictions in the literature.

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This paper is affectionately dedicated to the memory of the late Robert Emerson in whose laboratory this line of investigation was begun.

Literature Cited

1. Appleman, D. and Pyfrom, H. T. Changes in catalase activity and other responses induced in plants by red and blue light. Plant Physiol. 30: 543–549. 1955.
2. Arnon, D. I., Whatley, F. R. and Allen, M. B. Triphosphopyridine nucleotide reduction as a catalyst of photosynthetic phosphorylation. Nature 180: 182–185. 1957.
3. Arnon, D. I. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15. 1949.
4. Arnon, D. I. and Whatley, F. R. Is chloride the coenzyme of photosynthesis? Science 110: 554. 1949.
5. Arnon, D. I. and Whatley, F. R. Factors affecting oxygen production by illuminated chloroplast fragments. Arch. Biochem. 23: 141. 1949.
6. Arnon, D. I., Whatley, F. R. and Allen, M. B. Assimilatory power in photosynthesis. Science 127: 1026–1034. 1958.
7. Avron, M., Krogmann, D. W. and Jagendorf, A. T. The relation of photosynthetic phosphorylation to the Hill reaction. Biochim. Biophys. Acta. 30: 144–153. 1958.
8. Clendenning, K. A. and Gorham, P. R. Photochemical activity of isolated chloroplasts in relation to their source and previous history. Canadian Jour. Research C. 28: 114–139. 1950.
9. Clendenning, K. A. and Gorham, P. R. Photochemical activity of isolated spinach chloroplasts in relation to reaction conditions. Canadian Jour. Research C. 28: 78–101. 1950.
10. Davenport, H. E., Hill, R. and Whatley, F. R. A natural factor catalyzing reduction of methemoglobin by isolated chloroplasts. Proc. Roy. Soc. (London) B. 139: 346–358. 1952.
11. Granick, S. and Sager, R. Nutritional studies with *Chlamydomonas reinhardi*. Ann. New York Acad. Sci. 56: 831. 1953.
12. Gorham, P. R. and Clendenning, K. A. Anionic stimulation of the Hill reaction in isolated chloroplasts. Arch. Biochem. Biophys. 37: 199–223. 1952.
13. Hill, R. Oxygen evolved by isolated chloroplasts. Nature 139: 881–882. 1937.
14. Hill, R. Oxygen produced by isolated chloroplasts. Proc. Roy. Soc. (London) B. 127: 192–210. 1939.
15. Hill, R. Oxidoreduction in chloroplasts. Adv. Enzymol. 12: 1–39. 1951.
16. Hill, R. and Scarisbrick, R. The reduction of ferric oxalate by isolated chloroplasts. Proc. Roy. Soc. (London) B. 129: 238–255. 1939.
17. Holt, A. S. and French, C. S. The photochemical production of oxygen and hydrogen ion by isolated chloroplasts. Arch. Biochem. 9: 25–43. 1946.
18. Holt, A. S. and French, C. S. Oxygen production by illuminated chloroplasts suspended in solutions of oxidants. Arch. Biochem. 19: 368–378. 1948.
19. Jagendorf, A. T. The relationship between electron transport and phosphorylation in spinach chloroplasts. In: The Photochemical Apparatus. Brookhaven Symposia in Biology 11: 236–258. 1958.
20. Jagendorf, A. T. and Evans, M. The Hill reaction of red kidney bean chloroplasts. Plant Physiol. 32: 435–440. 1957.

21. JAGENDORF, A. T. and WILDMAN, S. G. The proteins of green leaves. VI Centrifugal fractionation of tobacco leaf homogenates and some properties of isolated chloroplasts. Plant Physiol. 29: 270–279. 1954.
22. KROGMANN, D. W. and JAGENDORF, A. T. Comparison of ferricyanide and 2,3,'6-trichlorophenol indophenol as Hill reaction oxidants. Plant Physiol. 34: 277–282. 1959.
23. KUMM, J. and FRENCH, C. S. The evolution of oxygen from suspensions of chloroplasts; the activity of various species and the effects of previous illumination of the leaves. Amer. Jour. Bot. 32: 291–295. 1945.
24. MACKINNEY, G. Absorption of light by chlorophyll solutions. Jour. Biol. Chem. 140: 315. 1941.
25. MARGULIES, M. and JAGENDORF, A. T. Loss of Hill reaction without loss of photosynthetic phosphorylation during dark storage of bean leaves. Plant Physiol. 33 Suppl.: xvii. 1958.
26. PUNNETT, T. Cell-free extracts of unicellular algae. A study of preparative techniques and reaction components. Doctoral thesis. University of Illinois, Urbana 1954.
27. PUNNETT, T. Some properties of algal chloroplast material. Revues des Fermentations. 1956.
28. PUNNETT, T. The pH optimum of the Hill reaction. Jour. Amer. Chem. Soc. 79: 4816. 1957.
29. PUNNETT, T. and FABIYI, A. Photochemical oxygen production from isolated algal chloroplast fragments. Nature 172: 947–948. 1953.
30. RABINOWITCH, E. Photosynthesis and Related Processes. Vol. II, part 2, chap. 37, B Interscience, New York 1956.
31. ROSIN, J. Reagent Chemicals and Standards. Pp. 162. Van Nostrand, New York 1955.
32. SPIKES, J. D., LUMRY, R., EYRING, H. and WAYRYNEN, R. Potential changes in suspensions of chloroplasts on illumination. Arch. Biochem. 28: 48–67. 1950.
33. WALKER, D. A. and HILL, R. Phosphorylation by illuminated chloroplast preparations. Biochem. Jour. 69: 57 P. 1958.
34. WESSELS, J. S. C. Investigations into some aspects of the Hill reaction. Doctoral thesis. University of Leyden, Leyden 1954.
35. WARBURG, O. and LÜTTGENS, W. In: Heavy Metal Prosthetic Groups and Enzyme Action. Oxford Press, Oxford 1949.

A PYRIDINE NUCLEOTIDE-CYTOCHROME C REDUCTASE ISOLATED FROM CHLOROPLASTS [1, 2]

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In 1957 Avron et al (1) extracted from spinach leaves a TPNH-diaphorase[3] which transfers electrons from TPNH to various oxidoreductive dyes but not to cytochrome c. Recently Marré et al (5, 6) isolated from chloroplasts of pea seedling a pyridine nucleotide-cytochrome c reductase which utilizes TPNH, markedly preferentially to DPNH, as an electron donor. The existence of these enzymes is of interest in view of the current concept among the investigators of photosynthesis that some cytochrome(s) as well as pyridine nucleotide(s) are involved in the mechanism of photosynthesis. Independently of these studies, we have been pursuing investigations on the oxidoreductive enzymes contained in chloroplasts, and from the chloroplasts of spinach and parsley an enzyme was isolated, which is similar to, but not identical with, that studied by Marré et al. The methods of isolation and purification as well as the properties of this enzyme form the subjects of this paper.

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[2] This research was supported by grants from the Ministry of Education and the Rockefeller Foundation.
[3] The following abbreviations are used: TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; FMN, flavine mononucleotide; FAD, flavine adenine dinucleotide; DPIP, 2,6-dichlorophenol indophenol; PCMB, p-chloromercuribenzoate; EDTA, ethylenediamine tetraacetate.

MATERIALS AND METHODS

The enzyme could be extracted from green leaves of spinach and parsley or chloroplasts isolated therefrom. To obtain chloroplasts, fresh leaves were homogenized in a Waring blendor for 3 minutes with the addition of 0.35 M NaCl solution. The homogenate was squeezed through cloth and centrifuged at 400 G for 5 minutes to remove the cell debris. The supernatant was centrifuged at 1700 G for 20 minutes, and the precipitate was collected, washed twice with 0.35 M NaCl solution by centrifugation, and used for further experiments. Two extraction procedures were employed. These led to the isolation of almost identical crude enzyme preparations.

PROCEDURE A: Fresh leaves or isolated chloroplasts (spinach) were ground in a mortar with quartz sand with the addition of chilled ammoniacal acetone-water (containing in volume percent: 98.5 acetone, 1.1 water and 0.4 ammonia) in a proportion of 85 ml per 100 g of fresh leaves or chloroplast suspension. The resulting mash was centrifuged at 1700 G for 15