THE BICARBONATE ION AND PHOTOSYNTHETIC OXYGEN EVOLUTION

BY

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THESIS

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LIST OF ABBREVIATION

chl	chlorophyll
DCMU	3- (3, 4 dichlorophenyl) -1, 1 dimethylurea
DCPIP	2, 6 dichlorophenol indophenol
DLE	delayed light emission
DPC	diphenyl carbazide
Q	primary electron acceptor from photosystem II
Z	primary electron donor to photosystem II

I. INTRODUCTION

In 1960 Warburg and Krippahl (1) discovered that the Hill reaction, *i.e.*, oxygen evolution by isolated chloroplasts illuminated in the presence of an oxidant such as ferricyanide, was dependent on the presence of CO_2. Warburg considered it a proof of his theory that oxygen, which is evolved during photosynthesis, arises from the splitting of CO_2 and not H_2O. Warburg's "proof" however, convinced very few. Rather than receiving serious consideration, Warburg's discovery seems to have been treated by most as a curiosity of questionable significance (2), or ignored entirely. That oxygen originates in water was, and still is, taken as fact. Perhaps for this reason the study of the "CO_2 effect" has since been sporadic and somewhat superficial.

At first, however, Warburg was not completely without supporters for his theory. Stern and Vennesland (3,4) repeated Warburg's results and showed that the CO_2 stimulation of ferricyanide reduction by chloroplasts is not due to the pH change which occurs when CO_2 dissolves in water. Furthermore the phenomenon is completely reversible. Adding back CO_2 to CO_2 depleted chloroplasts restores oxygen evolution. They tested chloroplasts from a number of species and found a CO_2 effect with all of them. These findings were enough to convince Stern and Vennesland of the merit of Warburg's views. As late as 1965 Vennesland, together with Olson and Ammeraal (5), published a paper in which Warburg's ideas were again put forward.

Aside from the previously mentioned authors, however, few others took Warburg's scheme seriously, including those who investigated, and confirmed, the CO_2 effect. Abeles *et al*. (6) repeated Warburg's results using kohlrabi but failed to see a CO_2 effect with sugar beet chloroplasts and concluded that the effect was not a general one. Izawa (7) showed that addition of carbonic anhydrase to the reaction media helped in developing CO_2 dependence. He also noted that CO_2 stimulation of oxygen evolution was greater at high light intensity (30,000 lux) than at low (3,000 lux). This last observation would seem to indicate that the CO_2 was involved in enzymatic rather than photochemical reactions since enzyme activity becomes rate limiting only at high intensity. Because the CO_2 effect he observed was small ($<$ 50 percent stimulation), Izawa discounted CO_2 as being important in the Hill reaction. Good (8) screened a number of organic and inorganic compounds searching for a substitute for CO_2 but was unsuccessful in finding one. The effect therefore appears specific to HCO_3^-. He also showed that high concentrations of chloride and acetate markedly increased the dependence of oxygen evolution on CO_2 as did uncouplers of phosphorylation. Though Warburg claimed that preillumination increased the CO_2 effect, Good was not able to confirm this. Good also published an intensity curve showing the CO_2 effect to be less at low light intensity, thus confirming Izawa (7).

Investigation of the CO_2 effect was extended by Punnett and Iyer (9) to include photophosphorylation. These workers again showed Hill reaction to be stimulated by CO_2. More interesting, however, was their discovery that phosphorylation was enhanced even more than electron flow, thus increasing the P/2e ratio. CO_2, in some fashion, couples electron flow to phosphorylation. They proposed that CO_2 was allowing the formation of some "high energy intermediate" to drive phosphorylation. The results of Punnett and Iyer were confirmed by Batra and Jagendorf (10). The latter showed, however, that although CO_2 increased phosphorylation and the P/2e ratio, the high energy intermediate, X_E, they (Batra and Jagendorf) measured was actually suppressed by CO_2. Besides concluding that X_E was not in fact an intermediate in the phosphorylation pathway, Batra and Jagendorf argued that there were two distinct effects of CO_2, that originally proposed by Warburg and Krippahl (1) related to oxygen evolution, and a second, discovered by Punnett and Iyer (9), related to the phosphorylation mechanism.

The latest study of the CO_2 effect, prior to the present work, was done by West and Hill (11) and reported in 1967. These authors showed a greater CO_2 effect with broken rather than whole chloroplasts. Of greater interest, however, were their light intensity curves. Measuring 2, 6-dichlorophenol indophenol (DCPIP) reduction, they found that the percent stimulation by CO_2 was independent of light intensity, an apparent contradiction to Good's (8) and Izawa's (7) results when oxygen evolution was measured. However, when West and Hill measured ferricyanide reduction, CO_2 dependence was observed only at high light intensity. This difference in low intensity CO_2 dependence, when different electron acceptors were used, was not adequately explained.

After reviewing the literature prior to 1967 then, one must admit that even though a number of workers have approached the problem of CO_2 dependence of the Hill reaction, very little real understanding or agreement resulted. While the CO_2 effect has been confirmed again and again, its significance was not apparent. The conditions for observing CO_2 dependence seemed to vary. The species active in the phenomenon, CO_2 or HCO_3^- was not known, nor was the site of action along the electron transport chain. Above all the mechanism by which CO_2 can stimulate Hill reaction could be, at best, the subject of poorly supported speculation. It was evident that a reinvestigation of the CO_2 effect was needed before any greater understanding of the phenomenon could come about. The purpose of the work reported in this thesis was, therefore, to establish conditions under which the CO_2 effect could be consistently observed, pinpoint the site of action of CO_2, and uncover as much as possible the mechanism by which CO_2 stimulates oxygen evolution.

II. MATERIALS AND METHODS

A. Chloroplast Isolation and Treatment

Maize (*Zea mays*; single cross hybrid GSC50) or oat (*Avena sativa* var. Cleland) plants were grown in vermiculite under artificial light (using a combination of 60W tungsten and 40W fluorescent lamps; 12/12 hour, light/dark photoperiod) at room temperature and harvested 8-14 days after planting. Just prior to harvesting, the plants were placed in a closed chamber and illuminated with white light using a GE 100W flood lamp for 1-2 hours. Nitrogen gas was passed through the chamber during illumination to make it relatively CO_2 free. This procedure consistently doubled the bicarbonate effect subsequently observed with the isolated chloroplasts and made the observation of a large (4-5 fold) effect much more consistent.

After light treatment, the harvested leaves were cooled in ice water and ground in Serval Omni-Mixer (15 sec. at 70 volts, 10 sec at 100 volts) at near freezing temperature using an isolation medium containing sucrose, 0.4M; NaCl, 0.01 M; and NaH_2PO_4/Na_2HPO_4 buffer, 0.05M, pH 6.8. The slurry was filtered through 4 layers of cheesecloth and the filtrate centrifuged for 1 minute at 200 xg. The supernatant fluid was poured off and centrifuged for 10 minutes at 1500xg. The resulting pellet was resuspended in a small amount of isolation medium, divided into portions and placed in a freezer. The frozen material was used usually within the following 2 weeks, during which time only a small loss of DCPIP reducing ability occurred.

To deplete the chloroplasts fragments of bound bicarbonate, they were thawed and suspended in a solution containing 0.25 M Na Cl, 0.04 M Na acetate, 0.05 M Na phosphate buffer, pH 5.0, which had been previously made CO_2 free by boiling and/or bubbling with N_2. The chlorophyll (chl) concentration was 50 μg/ml. A low pH of 5.0 was imperative for developing maximum bicarbonate dependence. With maize, only a small dependence developed if the chloroplasts were treated at a pH above 5.8, while below 5.0, Hill activity was seriously impaired. The chloroplast fragments remained 30 minutes in the dark at 15 to 17°C while N_2 was bubbled through the suspension. After 30 minutes, aliquots were drawn off with a syringe and placed in cold screw-capped test tubes previously flushed with N_2. The material was centrifuged, the supernatant poured off, and the tubes were placed in ice after once again being flushed with N_2. The bicarbonate-depleted chloroplasts were later resuspended in reaction mixture and assays were conducted.

The most critical aspects of the bicarbonate depletion procedure, noted above, are high anion concentration (chloride and acetate) to replace bound bicarbonate on the chloroplast, and low pH to facilitate the conversion of bicarbonate to dissolved CO_2 gas.

Further studies showed, however, that not all chloroplasts can tolerate a low pH. Chloroplasts from C_3 plants (e.g., oat, spinach (Spinacia oleracea), romain lettuce (Lactuca sativa) and, to a lesser extent, pokeweed (Phytolacca americana)) precipitate at pH 5 and are deposited on the sides of their container by the bubbling action of N_2 gas. Chloroplasts from C_4 plants which possess the dicarboxylic acid pathway in addition to Calvin cycle pathway for CO_2 fixation (e.g., maize, foxtail (Setaria viridis), purslane (portulaca oleracea) and Sorghum bicolor), on the other hand, show very little tendency to precipitate.[1] It is important to point out that even chloroplasts depleted of HCO_3^- at higher pH (5.8 - 6.0) to prevent precipitation, showed some increase in either ferricyanide reduction or oxygen evolution when provided with HCO_3^-.

Glutaraldehyde-fixed chloroplasts were prepared by the method of Park (12). To check fixation, glutaraldehyde treated and untreated chloroplasts were subjected to osmotic shock and observed under the microscope. The unfixed chloroplasts were swollen, had poorly defined borders, were pale green in color and showed no subchloroplast structure. Fixed chloroplasts were compact, had clearly defined edges, were dark green and had a "grainy" appearance. The glutaraldehyde-fixed chloroplasts were then depleted of bicarbonate in the same manner as described above.

To prepare chloroplasts for the experiment in which the artificial electron donor diphenyl carbazide (DPC) was used (13), they were placed in a water bath at 49°C for 5 minutes (14) immediately after treatment to deplete them of bicarbonate. This heat treatment destroyed the

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[1] It seems there may be some difference between these two groups of plants in the structural protein of their thylakoids causing one to precipitate under more acid conditions. Though this apparent difference in chloroplasts from C_3 and C_4 plants has not, to our knowledge, been previously reported, further verification and pursuit of the phenomenon was considered not within the scope of this investigation.

chloroplasts' ability to reduce DCPIP without added electron donors except for trace amounts in the presence of bicarbonate. After heat treatment the chloroplasts were collected by centrifugation and used as usual.

B. Assay Procedures

1. Dichlorophenol Indophenol Reduction

DCPIP reduction was measured using a Cary 14 recording spectrophotometer equipped with a side attachment to illuminate the sample. The actinic beam from a GE 120V, 650W, DVY lamp passed through a Corning C.S. 2-59 red cut-off filter, emerging with an intensity of 2×10^6 ergs cm^{-2} sec^{-1}. An appropriate interference filter (λ max, 597 nm; half band width, 12 nm) was placed in front of the photomultiplier to block out the actinic light so the change in absorbance of the sample, measured at 597 nm wavelength, could be monitored as it occurred. The reaction cuvette was flushed with N_2 and sealed, keeping the conditions initially anaerobic. Solutions were transferred from otherwise sealed vessels by means of syringes to minimize atmospheric contamination.

All reaction mixtures were buffered and those to which a high concentration of bicarbonate was added (causing an increase in pH) were brought back to the desired pH by the addition of dilute HCl. Solutions containing bicarbonate were kept sealed to prevent loss of CO_2 gas.

The rate of DCPIP reduction was taken from the slope of the recorder trace starting with the onset of actinic illumination. These readings were converted into μmoles DCPIP reduced by using the extinction coefficient published by Armstrong (15) at pH 6.8 and relative coefficients measured under our own conditions at other pHs. At very low bicarbonate concentrations, a definite lag period, lasting a variable number of seconds (1-15) was sometimes observed. When it occurred, the reaction rate was taken from the extent of the absorbance change at one minute after the onset of illumination. Thus the lag does not play a significant role in the overall effect discussed here. In the extreme case of a 15 second lag in the bicarbonate free system, the initial rate of DCPIP reduction, once it began, was still about a fourth of the rate seen at high concentrations of bicarbonate. To obtain consistent results at low HCO_3^- concentrations, it was necessary to assay quickly after suspending the chloroplasts in reaction mixture.

2. Ferricyanide Reduction: Amperometric Method

Initial rates of ferricyanide reduction were measured using a platinum rate electrode similar to that described by Joliot and Joliot (16) but used in the unmodulated mode. The platinum electrode was polarized at + 0.7 volts relative to the Ag/AgCl electrode. Thus it measured ferrocyanide production.

Chloroplasts were placed on the platinum surface and covered with a dialysis membrane. Through the chamber above this membrane flowed CO_2 free solution containing 0.25 M NaCl, 0.04 M Na acetate, 1 mM potassium ferricyanide and 0.05 M Na phosphate, pH 6.8. Flow through this chamber could be switched to permit entry of the same solution plus 0.01 M $NaHCO_3$. Above this chamber, separated by another membrane, was a second chamber housing the Ag/AgCl electrode. Through this chamber flowed solution free of Hill oxidant.

The sample was illuminated with white light (approximately 10^6 ergs cm^{-2} sec^{-1}) from a tungsten ribbon filament lamp. The beam passed through a heat filter, focusing lens, shutter, and buffer (path length, 2 cm) before striking the sample. Assays were conducted using 0.5 second shutter openings. The signal generated at the electrode was amplified by a keithley microvolt ammeter (model 150B) and recorded, on a Heath Servo recorder (model 1 EUW-20A) as a "spike" on the chart paper. At low light intensity the spike height was linear with intensity, allowing a good means of measuring relative quantum yields of ferricyanide reduction. Light intensity was reduced from saturation by means of neutral density filters.

After placing the chloroplasts on the electrode, several minutes were allowed for settling, after which the ability to reduce ferricyanide was assayed, in the absence of exogenous HCO_3^-, by means of several half-second flashes of light given at 30 second intervals. The flow of buffer over the chloroplasts was then switched to that containing HCO_3^- and several more half-second assays were conducted. The effect of pretreatments in enhancing the stimulation seen when HCO_3^- was added could then be observed.

3. Ferricyanide Reduction: Spectral Method

Ferricyanide reduction was measured as an absorbance change at 420 nm using a Cary 14 recording spectrophometer equipped with a side attachment to illuminate the sample. For both oxygen evolution (see below) and ferricyanide reduction measurements actinic illumination came from GE 120V, 650W, DVY lamps. The beams passed through a 15 cm thick layer of water and Corning C.S. 3-71 yellow cut-off filters before emerging with intensities of 5 x 10^5 ergs cm^{-2} sec^{-1}. When simultaneous measurements of oxygen evolution and ferricyanide reduction were made, chloroplasts were suspended in reaction mixture and then aliquots were drawn off and placed in the respective instruments. Thus oxygen evolution and ferricyanide reduction could be compared using identical samples.

4. Oxygen Evolution

Net oxygen evolution was measured by a Clark-type electrode setup using a Yellow Springs Oxygen Monitor (model 53). The signal was recorded by an Esterline Angus (model E11015) recorder.

5. Chlorophyll a Fluorescence Measurements

Fluorescence transient measurements were made as described by Munday and Govindjee (17). The photomultiplier signal was fed through a Tektronix oscilloscope (type 502) and displayed on an Esterline Angus (model E 11015) or a Midwestern oscillographic recorder (model 801). Chl *a* fluorescence was excited with blue-green light (Corning C.S. 4-96 and 3-73 filters). The light intensity was 8 x 10^4 ergs cm^{-2} sec^{-1} or lower, reduced by means of calibrated neutral density filters. Fluorescence was observed at 685 nm (half band width, 6.6 nm) through a Bausch and Lomb monochromator (blazed at 750 nm). A Corning C.S. 2-58 filter, placed at the entrance slit of the analysing monochromator, eliminated stray exciting light. Recovery of the fluorescence transient in the dark was determined as described by Mohanty *et al.* (18).

6. Delayed Light Emission Measurements

Measurements of delayed light emission (DLE) were made using the instrument described by Jursinic and Govindjee (19). For these measurements, chloroplast suspensions were preilluminated with blue light (Corning C.S. 4-96 filter). The intensity of exciting light was 400 ergs cm^{-2} sec^{-1}, or 8000 ergs cm^{-2} sec^{-1} (in some experiments).

Other details of the methods are described in the appropriate chapters, and legends of figures and tables.

III. BICARBONATE STIMULATION OF FERRICYANIDE REDUCTION, OXYGEN EVOLUTION AND PHOTOINACTIVATION

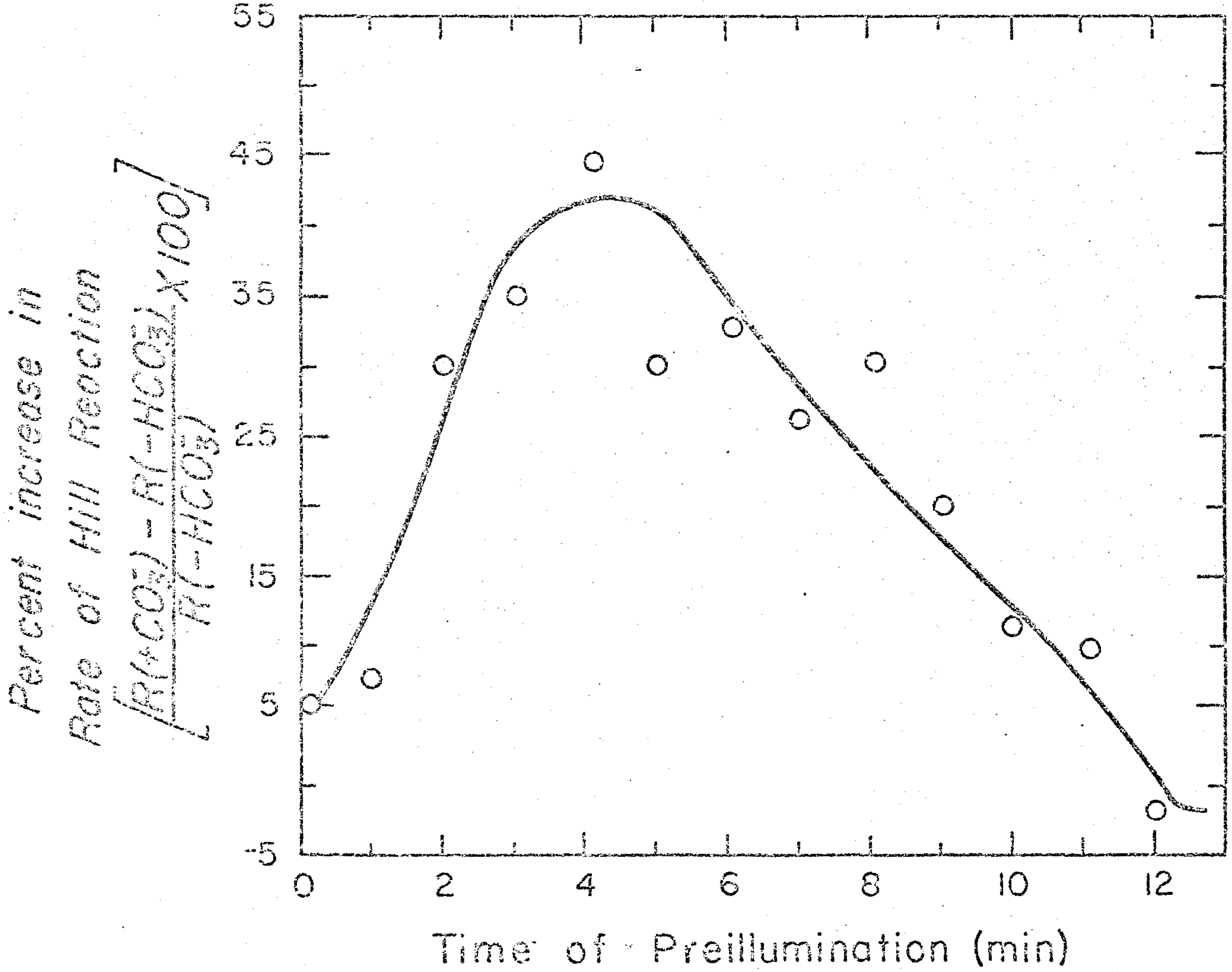
A. Increase in HCO_3^- Dependence with Preillumination

The role of preillumination in increasing HCO_3^- dependence is a matter of controversy. Warburg and Krippahl (1), the discoverers of the HCO_3^- effect, claimed that preillumination was necessary to maximize HCO_3^- dependence. Good (8) preilluminated chloroplasts in the absence of ferricyanide and found no increased dependence on HCO_3^-. We have found that preillumination of chloroplasts in HCO_3^- free medium, but in the presence of ferricyanide, leads to increased dependence of the reaction on HCO_3^-.

Oat chloroplasts not previously depleted of HCO_3^- were placed on the platinum rate electrode. They were permitted to perform Hill reaction; i.e., they were illuminated, in the presence of ferricyanide, for a variable length of time while HCO_3^- free solution passed over the membrane holding them to the surface of the platinum. After this period of illumination, several half-second assays were conducted. The chloroplasts were then given HCO_3^- containing solution and several more half-second assays were performed.

Dependence of Hill reaction on HCO_3^- as a function of preillumination time is shown in Figure 1. The first four minutes of preillumination cause a progressive increase in HCO_3^- dependence. After this time, dependence again decreases to essentially zero between 10 and 12 minutes. Dark controls showed no change in dependence on HCO_3^-. Likewise illuminating chloroplasts in the absence of ferricyanide did nothing to increase the dependence of Hill reaction on HCO_3^-. In this respect we confirm the

Figure 1. Percent increase in the rate of ferricyanide reduction with added HCO_3^- as a function of preillumination time. The solution passing over the electrode membrane contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate, pH 6.8, 0.5 mM potassium ferricyanide ± 0.01 M Na HCO_3^-. Saturating white light (10^6 ergs cm^{-2} sec^{-1})used during preillumination and half-second assays ; anaerobic conditions. Oat chloroplasts (43 μg chl/ml in stock) ; Average of two experiments.


Percent increase in
Rate of Hill Reaction
$\left[\frac{R(+CO_3^-) - R(-HCO_3^-)}{R(-HCO_3^-)} \times 100\right]$
55
45
35
25
15
5
-5
0
2
4
6
8
10
12
Time of Preillumination (min)

results of Good (8) who also showed that preilluminating chloroplasts in the absence of ferricyanide did not lead to greater HCO_3^- dependence of the Hill reaction. Good, however, did not attempt to observe increased HCO_3^- dependence with preillumination in the presence of ferricyanide. It appears, therefore, that increased HCO_3^- dependence of the Hill reaction is a function of electron flow rather than light absorption alone. It should be pointed out that the decline in HCO_3^- dependence after 4 minutes is associated with a decline in the over-all activity, which is reduced to nearly zero after 10-12 minutes.

It should also be mentioned that the results presented in Figure 1 were most reproducible when chloroplasts were frozen for at least a week. Fresh material, or that frozen less than about one week, also showed increasing dependence of Hill reaction on exogenous HCO_3^- for the first 4 minutes of preillumination, thereafter for both, over-all activity and HCO_3^- dependence were maintained for much longer, declining to zero only after about 20 minutes. Prolonged freezing may be causing a slow destruction of membrance integrity accounting for the more rapid loss of activity and associated decrease in HCO_3^- sensitivity.

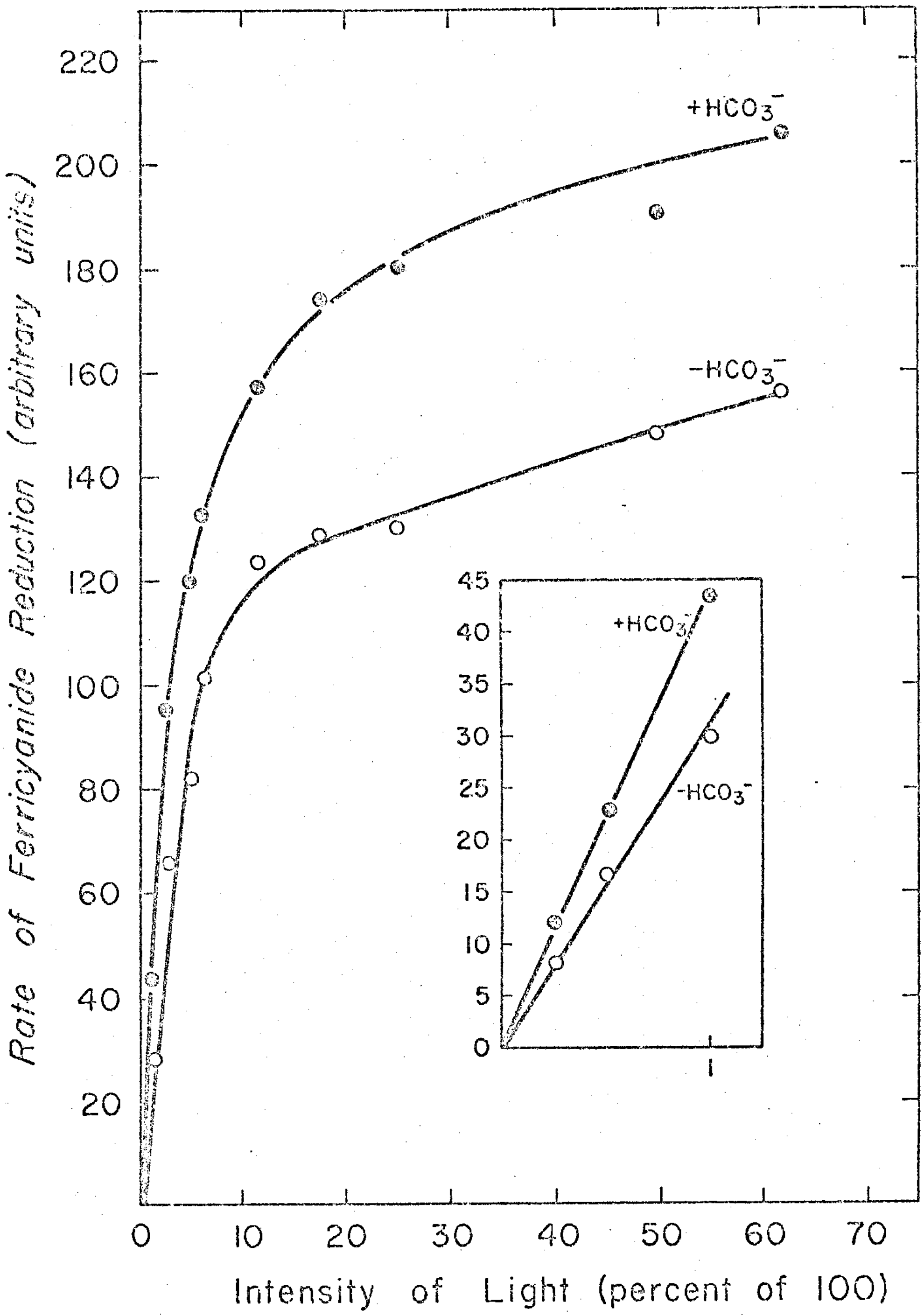
Since HCO_3^- not "used up" during Hill reaction (20) (which would imply permanent incorporation into an organic molecule) an increase in HCO_3^- dependence with time during Hill reaction must result in some other way. We suggest that HCO_3^- is initially bound, perhaps directly to reaction centers, or exists in some complexed condition. As Hill reaction proceeds, HCO_3^- becomes unbound, or otherwise free (as CO_2?). This "free" HCO_3^- may be recycled to some extent within the thylakoids, but increasing dependence of the reaction on exogenous HCO_3^- with time implies that at least some is lost. It is also clear from the fact that HCO_3^- dependence decreases after 4 minutes of illumination that at least one other factor limits the reaction. This factor, usually described by the general term of photoinactivation, is not well understood (21, 22, 23).

B. Bicarbonate Effect as a Function of Light Intensity

It is important to know whether or not HCO_3^- can stimulate Hill reaction at low light intensities (where the rate of the reaction increases linearly with increase in light intensity) since a low intensity effect would indicate an involvement in photochemical reactions rather than in purely enzymatic ones. Unfortunately, the published data appear conflicting on this point (7,8,11). We, therefore, reinvestigated this problem and found that the dependency of ferricyanide reduction on exogenous HCO_3^- is observed even at low light intensities, the magnitude of HCO_3^- effect being independent of light intensities used.

Oat chloroplasts, again not previously depleted of HCO_3^-, were placed on the platinum rate electrode and allowed to perform Hill reaction in the absence of exogenous HCO_3^- for 4 minutes. This induced a degree of dependence of HCO_3^- as shown in figure 1. Half-second assays were then conducted without, and then with HCO_3^-, as described previously. In this case light intensity was varied using calibrated neutral density filters. Figure 2 shows that the bicarbonate effect seen under these experimental conditions is independent of light intensity. Even at the lowest intensity (in the linear portion of the light curve) where ferricyanide reduction could still be accurately measured

Figure 2. Rate of ferricyanide reduction with and without 0.01 M $NaHCO_3$ as a function of light intensity. Oat chloroplasts preilluminated 4 min. in saturating white light; light intensity varied with calibrated neutral density filters; average of 3 series; other conditions as in Figure 1.


Rate of Ferricyanide Reduction (arbitrary units)
220
200
180
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
Intensity of Light (percent of 100)
+HCO$_3^-$
-HCO$_3^-$
45
40
35
30
25
20
15
10
5
0
1
+HCO$_3^-$
-HCO$_3^-$

(see insert), HCO_3^- stimulated the activity to the same degree as at saturating intensity. This is shown by the constancy of the ratio of the rate of Hill reaction in the presence to that in the absence of HCO_3^- as a function of light intensity. These results differ with those published by Good (8) who measured oxygen evolution manometrically and showed less stimulation of HCO_3^- at low intensity than at high. However, West and Hill (11), measuring DCPIP and ferricyanide reduction spectrophotometrically, showed that with DCPIP reduction the HCO_3^- effect was independent of light intensity but with ferricyanide reduction, dependence on HCO_3^- was less at low intensity. We feel that our results are more reliable for several reasons: a) since assays consisted of half-second flashes of actinic light, the data more aptly reflect the ability of HCO_3^- to stimulate initial rates of ferricyanide reduction at the various intensities; b) the same sample was used without, and then with HCO_3^-, at each intensity; and c) the method of assay used in our work here, is more sensitive and accurate, especially at low intensities, than spectrophotometric measurements since ferricyanide has a fairly low absorbtivity even at the 420 nm wavelength where is is usually measured.

We conclude from the results in Figure 2 that HCO_3^- is involved in early "photochemical reactions" of photosystem II rather than enzymatic reactions somewhat removed from the reaction centers.

C. Comparison of the Effect of HCO_3^- on Oxygen Evolution and Ferricyanide Reduction

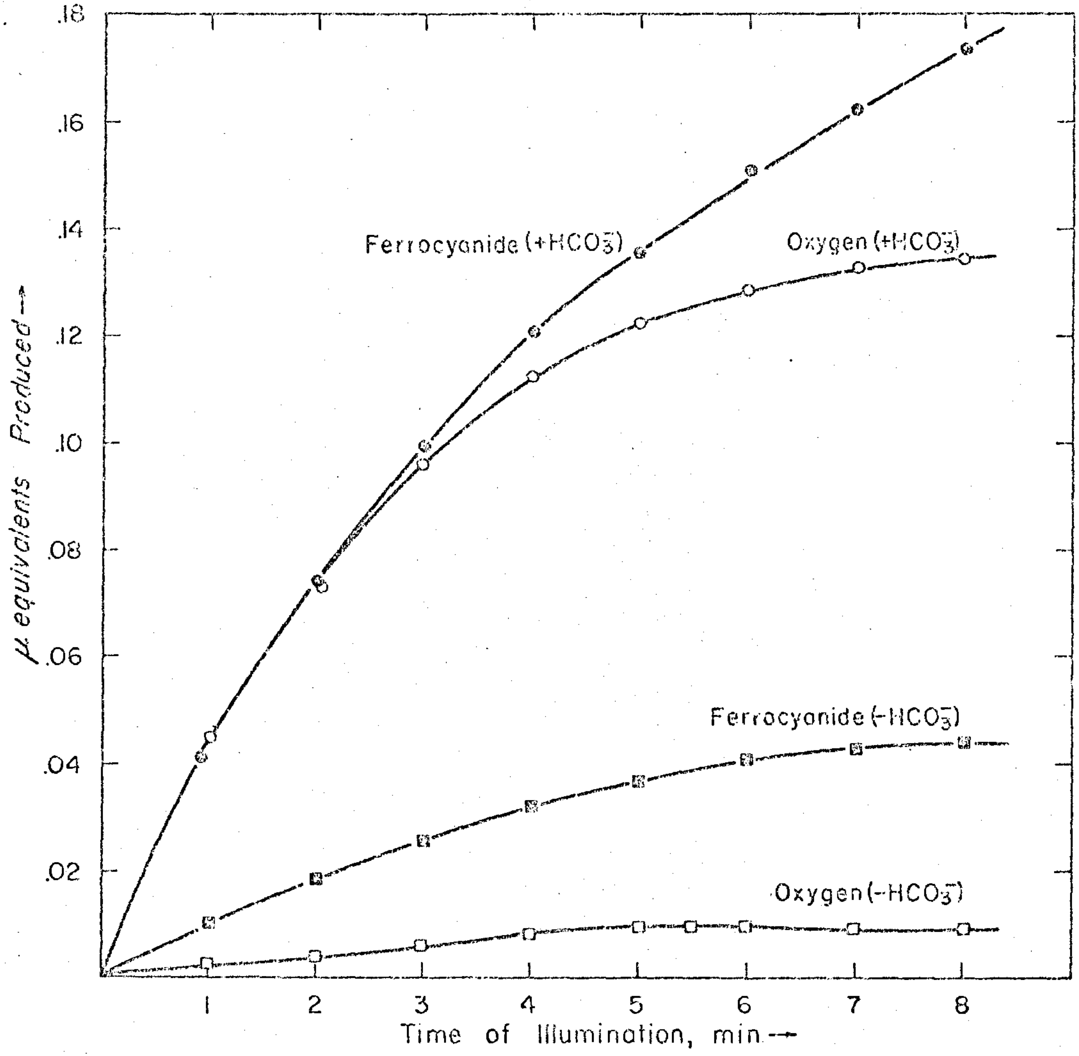
Good (8) has reported observing an excess of 10 fold stimulation of oxygen by HCO_3^- addition. However, those who have measured DCPIP or ferricyanide reduction failed to observe more than a 4-5 fold stimulation (10, 11). To clarify these apparent discrepancies, we measured oxygen evolution and ferricyanide reduction in identical samples and found that oxygen evolution is indeed more dependent on HCO_3^- than ferricyanide reduction, as shown in Figure 3.

When maize chloroplasts are depleted of HCO_3^-, their oxygen evolving activity is reduced by more than 90 percent. However their ferricyanide reducing ability is suppressed by less than 80 percent. In the presence of HCO_3^-, in contrast, equal μ equivalents of oxygen and ferricyanide are produced, at least during the first several minutes of illumination. That is, in the presence of HCO_3^-, for every molecule of oxygen evolved four electrons are transferred to ferricyanide.

Figure 4 (replot of Figure 3) shows the magnitude of the differential effect of HCO_3^- on oxygen evolution and ferricyanide reduction. HCO_3^- can increase ferricyanide reduction 4-5 fold whereas oxygen evolution, in the same samples, is increased over 15 fold.

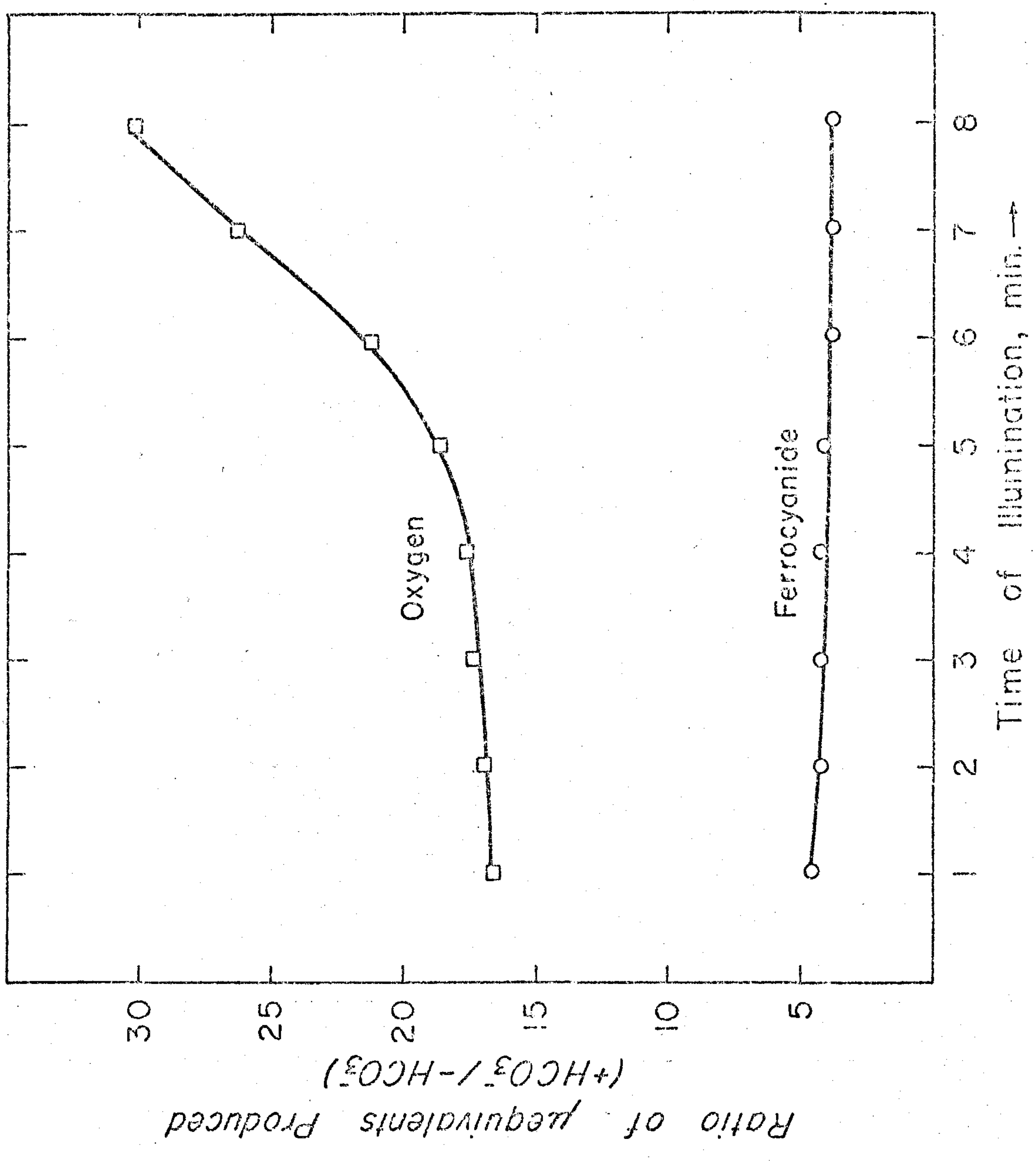
It may be possible to explain the differences in O_2 evolution and ferricyanide reduction by assuming greater O_2 uptake in the absence of HCO_3^- than in the presence of this ion. If the product of oxygen uptake is hydrogen peroxide, (H_2O_2), two molecules of O_2 can be taken up for every one evolved. One can imagine a situation in which O_2 uptake were exactly equal to O_2 evolution. In such a situation, half of the electrons generated by oxygen evolution would not be forming H_2O_2 and could therefore be used to reduce ferricyanide. Thus one could observe ferricyanide reduction accompanied by zero net oxygen evolution. However, if this were the mechanism of action of HCO_3^-, i.e. preventing O_2 uptake, one might also expect to observe an effect in the absence of ferricyanide, greater O_2 uptake ought to be observed in the absence of HCO_3^- than in the presence of this ion. Under my

Figure 3. Comparison of oxygen evolution and ferricyanide reduction in the presence and absence of HCO_3^-. Reaction mixtures contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate, pH 6.8, 1 mM potassium ferricyanide ± 0.01 M $NaHCO_3$ and 15 μg of chl/ml of maize chloroplast suspension. Light intensity, 5×10^5 ergs cm^{-2} sec^{-1}; Corning C.S. 3-71 yellow cut-off filter; initially, anaerobic conditions.



.18
.16
.14
.12
.10
.08
.06
.04
.02
μ equivalents Produced →
1
2
3
4
5
6
7
8
Time of Illumination, min →
Ferrocyanide (+HCO_3^-)
Oxygen (+HCO_3^-)
Ferrocyanide (-HCO_3^-)
Oxygen (-HCO_3^-)

Figure 4. Bicarbonate effect (ratio of rates with and without HCO_3^-) on oxygen evolution and ferricyanide reduction in identical samples. This represents a replotting of data presented in Figure 3.


Oxygen
Ferrocyanide
Time of Illumination, min. →
1
2
3
4
5
6
7
8
Ratio of μequivalents Produced
(+HCO$_3^-$/-HCO$_3^-$)
30
25
20
15
10
5

(initially anaerobic) conditions, however, I was not able to see any difference in O_2 uptake in the presence of absence of HCO_3^- when ferricyanide was omitted from the reaction mixture (data not shown). Even more unexpectedly, West and Hill ( ) report that HCO_3^- stimulates rather than inhibits oxygen uptake in the absence of exogenous electron acceptors. Therefore, preventing O_2 uptake does not seem to be the mechanism of action of HCO_3^- under all conditions, but may still be considered under the conditions of this experiment.

The differences in O_2 evolution and ferricyanide reduction can also be interpreted to mean that there must exist a fairly substantial amount of an endogenous electron donor capable, under the conditions of these experiments of donating electrons to photosystem II without evolving oxygen. This will account for the greater equivalents of ferricyanide reduced than oxygen evolved from HCO_3^- depleted chloroplasts. This interpretation is consistent with other available data. Residual ferricyanide reduction in the absence of oxygen evolution was first observed by Kahn (24), using a protein-chlorophyll complex isolated from chloroplasts after treatment with Triton X-100. Kutyurin _et_ _al_. (25), using chloroplasts and measuring oxygen evolution and ferricyanide reduction simultaneously, found that oxygen evolution was more inhibited by treatment with low concentrations of dodecylsulfate, dignitonin and CMU than was ferricyanide reduction. Most recently, Huzisige and Yamamoto (26), using photosystem II particles, found residual ferricyanide reduction without oxygen evolution. These last authors showed that the ferricyanide reduction was not simply an apparent absorbance change caused by pigment photobleaching. No significant absorbance change was seen when ferricyanide was omitted from the reaction mixture during illumination. (Our control experiments gave similar results.) In addition these authors used colorimetric methods to confirm that ferricyanide was, in fact, reduced under conditions where no oxygen was evolved.

In light of the work of Yamashita et al. (27), however, the question of pigment photobleaching may still be raised. These authors have shown that carotenoids are irreversibly photobleached when chloroplasts are illuminated in the presence of ferricyanide and certain inhibitors of the Hill reaction but not in the absence of these substances. The absorbance change at 420 nm due to photobleaching is small (approximately 25 percent) relative to the change seen at 500 nm. In our control experiments we also see a small change in absorbance at 500 nm during illumination indicating some pigment bleaching. However, the amount of change at 500 nm is always less than 30 percent of the absorbance change at 420 nm. We therefore estimate that photobleaching can account for less than 10 percent of the absorbance change we observe at 420 nm. The remaining change must be due to ferricyanide reduction.

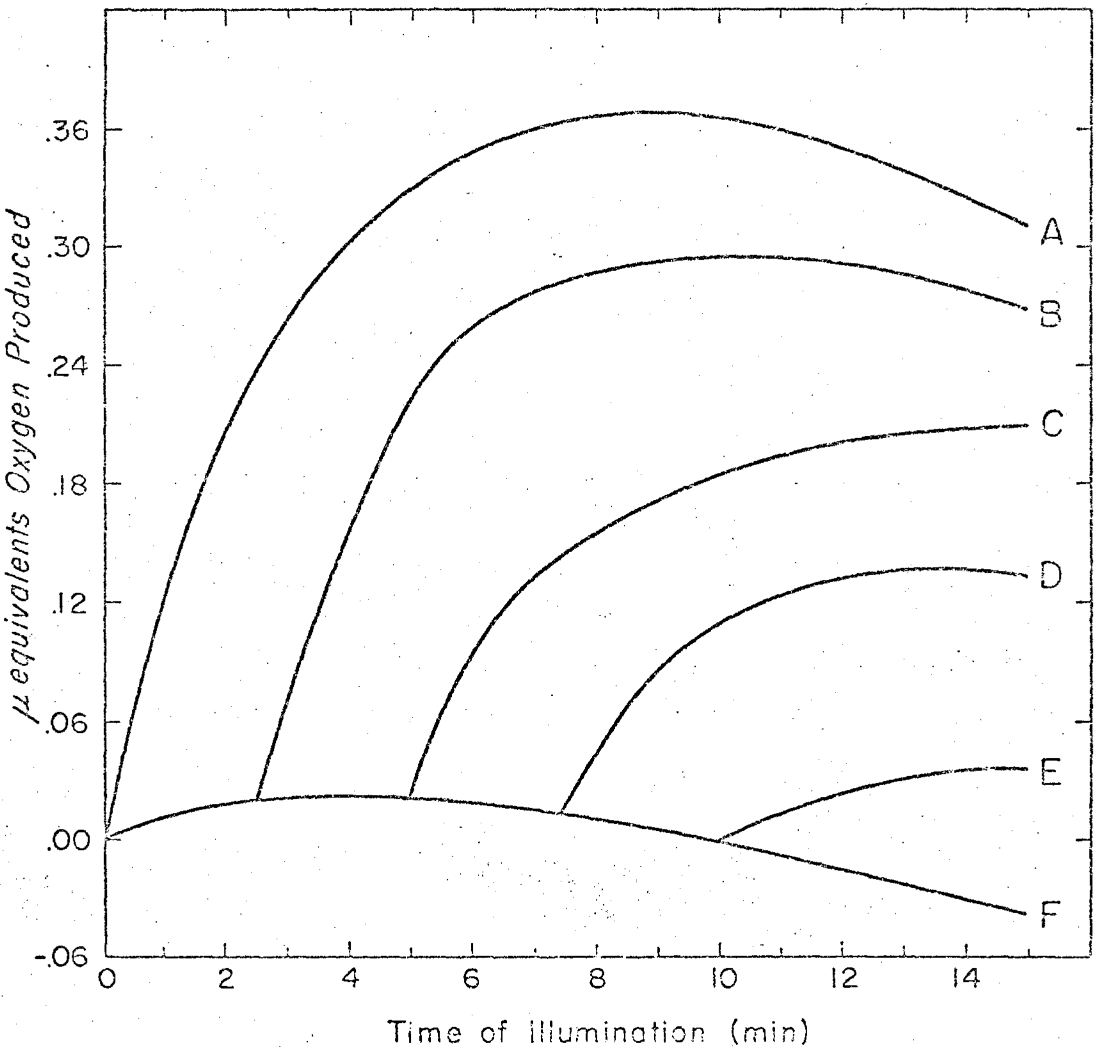
Despite considerable evidence of its existence, the identity of this endogenous electron donor is unknown. It is difficult even to imagine what could exist in such large amounts within the thylakoid membrane or photosystem II particles. Still another interesting consideration is the possible role of this donor in the functioning of photosystem II and also its relationship to HCO_3^-. Our data in Figure 3 can be explained by postulating that HCO_3^- stops the "abnormal" flow of electrons from this endogenous donor, switching the flow so that electrons come from the oxygen evolving system. Another possibility is that HCO_3^- and this unidentified donor actually cooperate in some fashion to evolve oxygen. Thus both may be necessary for oxygen evolution. One may speculate that photoinactivation could be due, at least, in part, to the depletion of this required substance which can, under certain circumstances such as HCO_3^- depletion, act as an electron donor. This idea will be later substantiated (see discussion of Figure 6).

D. The Effect of HCO_3^- Injected after Different Periods of Illumination

Maize chloroplasts were depleted of HCO_3^- and illuminated in the presence of ferricyanide. After different periods of time, illumination was stopped, HCO_3^- was injected and illumination resumed after 2 minutes. The initial rates of oxygen evolution and the net amounts of oxygen evolved are shown in Figure 5. Curve F resulted from illuminating chloroplasts for 15 minutes in the absence of HCO_3^-. Curves A, B, C, D, and E were obtained by injecting HCO_3^- at 0, 2.5, 5, 7.5 and 10 minutes into different samples respectively. (The two minutes dark intervals, required to achieve a flat baseline after injection of the HCO_3^- solution, were omitted in redrawing the traces.)

This family of curves (A-E) show that as HCO_3^- injection is delayed, the net amount of oxygen evolved and the initial rate of evolution, after injection, declines. For example, if HCO_3^- injection is delayed 7.5 minutes, both the initial rate of oxygen evolution, and the maximum amount of oxygen evolved, are reduced about 60 percent. Thus it is clear that photoinactivation is taking place in the absence of HCO_3^- and in the absence of substantial amounts of net oxygen evolution. Further data on photoinactivation including dark controls will be discussed below.

Figure 5. Oxygen evolution in response to (10 mM) HCO_3^- injected into different samples after varying periods of illumination. The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate, pH 6.8, 2 mM potassium ferricyanide and 40 µg of chl/ml of maize chloroplast suspension. Curves A, B, C, D, and E represent oxygen generated after injection of 10 mM (final concentration) HCO_3^- to 0.01 M at 0, 2.5, 5, 7.5 and 10 minutes respectively. Chloroplasts not given HCO_3^- at all generated oxygen as represented by curve F. The two minutes dark interval, required to achieve a flat baseline after injection of HCO_3^- solution, was omitted in redrawing the traces. Other conditions were the same as in Figure 3.

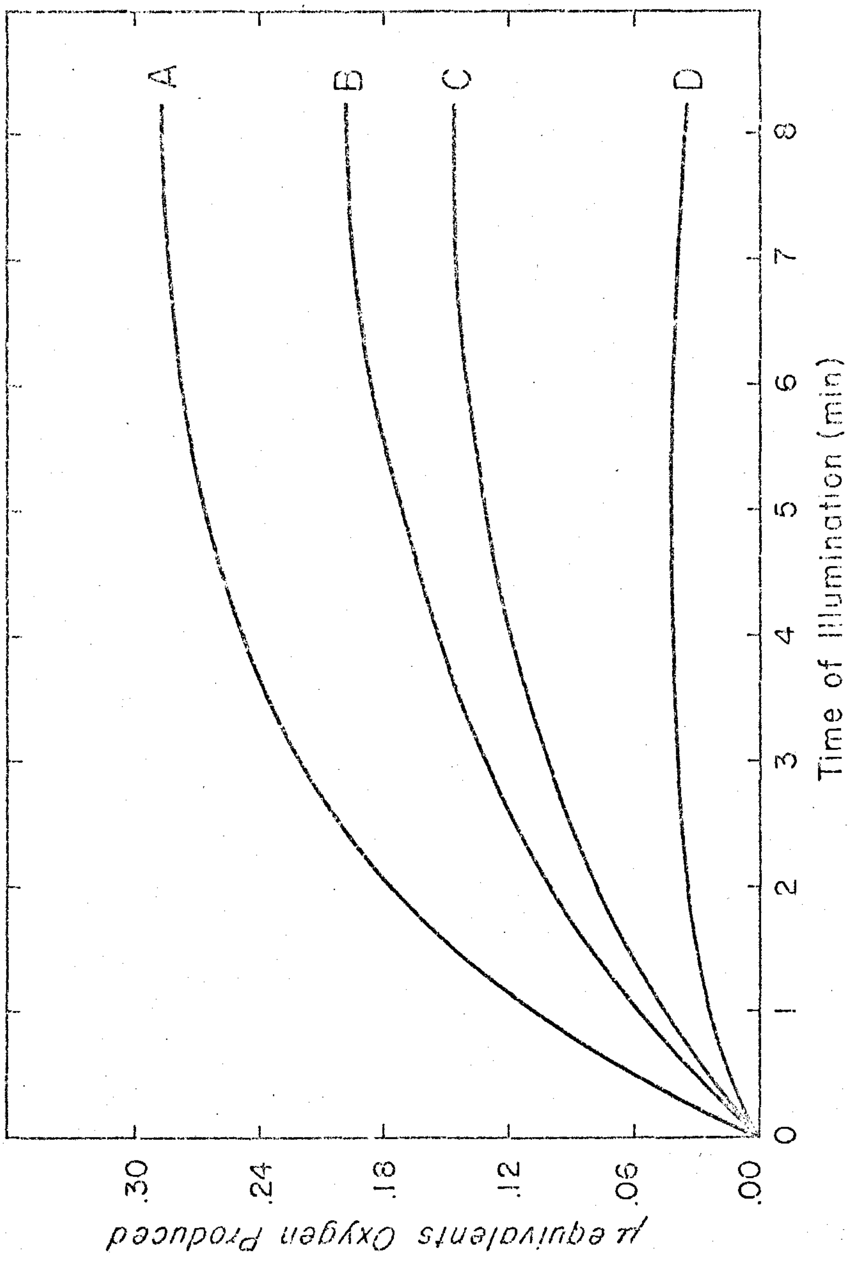

.36
.30
.24
.18
.12
.06
.00
-.06
μ equivalents Oxygen Produced
0
2
4
6
8
10
12
14
Time of Illumination (min)
A
B
C
D
E
F

E. The Effect of HCO_3^- on Photoinactivation of Chloroplasts

Chloroplasts depleted of HCO_3^- were illuminated with saturating light for 8 minutes under various conditions. Residual oxygen evolving ability was then assayed during a second 8 minute period. During the assay period both ferricyanide and HCO_3^- were present in all samples. The results are shown in Figure 6. Curve A shows the remaining activity in the dark controls. These chloroplast samples were kept 8 minutes in the dark in the presence of ferricyanide and either with or without HCO_3^-. Curve D shows the residual activity in chloroplasts which were illuminated 8 minutes in the presence both ferricyanide and HCO_3^-. After this treatment, the oxygen evolving activity is reduced by 85 percent. Curve C shows the activity remaining after 8 minutes of illumination in the presence of ferricyanide but in the absence of HCO_3^-. In this case activity was reduced 50 percent. Curve B was obtained using chloroplast samples illuminated 8 minutes in the absence of ferricyanide, some with, others without, HCO_3^-. These treatments reduced activity only about 30 percent.

We can draw several conclusions from these data concerning both the HCO_3^- effect and photoinactivation. Clearly HCO_3^-, which markedly increases electron flow coupled to oxygen evolution, also stimulates photoinactivation (Curve D). Illumination in the presence of ferricyanide alone ($-HCO_3^-$) also leads to somewhat less photoinactivation though very little oxygen is evolved (see Figure 3 and 5). It should be kept in mind that electron flow is taking place under this condition even if it is not necessarily coupled to oxygen evolution (Figure 3).

Figure 6. Residual oxygen evolution, assayed during an 8 minute period, after various pretreatment. During the assays shown here all samples contained the same reaction mixture as in Figure 5. Illumination conditions were the same as in Figure 3. Initially, anaerobic conditions. The pretreatments were: (A): 8 minutes dark, + ferricyanide ± 10 mM HCO_3^-; (B): 8 minutes saturating light, - ferricyanide ± HCO_3^-; (C): 8 minutes saturating light, + ferricyanide, - HCO_3^-; (D): 8 minutes saturating light, + ferricyanide, + HCO_3^-.


μ equivalents Oxygen Produced
.00
.06
.12
.18
.24
.30
Time of Illumination (min)
0
1
2
3
4
5
6
7
8
A
B
C
D

Photoinactivation then, under these experimental conditions, seems more a function of the amount of electron flow (from whatever source) than the amount of oxygen evolved.

Since all of the above-mentioned treatments were done under initially near-anaerobic conditions, we thought it might be possible that the increased photoinactivation seen in the presence of HCO_3^- might be due to an indirect effect of the increased amount of oxygen which develops under this condition (see Figures 3 and 5). However, it was found that increasing the oxygen concentration in samples by opening the reaction vessel to the air momentarily, illuminated in the absence of HCO_3^- did not appreciably increase photoinactivation.

Samples which were illuminated in the absence of ferricyanide also showed some photoinactivation (Curve B, Figure 6). This amount of photoinactivation is not considered to be due to some direct irreversible damage by light rather than an effect of electron flow. Such direct damage is associated with much higher light intensities (28) than were used in these experiments. Moreover it is known that a considerable amount of oxygen uptake can be measured when chloroplasts are illuminated in the absence of a Hill oxidant (29, 30, 31). We also monitored oxygen during illumination of chloroplasts in the absence of ferricyanide and observed net oxygen uptake, even in samples made almost completely anaerobic before illumination. This net oxygen uptake of the trace amounts of oxygen remaining is about the same in the presence or absence of HCO_3^-. It is probable then that at least some, and perhaps all, of the "photoinactivation" reported here in the absence of a Hill oxidant can still be correlated to electron flow rather than to some direct damage by light.

Photoinactivation has been shown by Satoh (22, 23) to be a complex phenomenon, different for photosystems I and II. The usual way of explaining photoinactivation of photosystem II is to assume the accumulation of reduced intermediates which react with, and damage, the system itself. If this were the case, one might expect that providing an electron sink such as ferricyanide would prevent the accumulation of these reduced intermediates and hence retard photoinactivation. (Ferrocyanide itself, rather than damage chloroplasts, has been shown by Brewer and Jagendorf (32) to have a protecting effect.) This is not the case. Greater damage is seen in the presence of ferricyanide. From this data, I suggest that photoinactivation of photosystem II may, be due, in part, to the depletion of a pool of some substance required for the functioning of the oxygen evolving side of the photosystem. This substance may be the same one which appears as a nonoxygen evolving electron donor in the absence of HCO_3^-. This hypothesis also implies that this unidentified material is used up even more rapidly in the presence of HCO_3^- since this ion accelerates photoinactivation.

IV. CARBON DIOXIDE OR BICARBONATE AS THE ACTIVE MOIETY; SITE OF ACTION

A. The Active Form of CO_2 in Stimulating the Hill Reaction

Experiments were conducted to determine whether dissolved CO_2 gas or bicarbonate ion is the active species in stimulating Hill reaction. A bicarbonate concentration study was done at pH 5.8 and 6.8. If dissolved CO_2 was the active form, we would expect that a subsaturating concentration would show a larger stimulation of the Hill reaction at pH 5.8 than an pH 6.8. (Since the pk_a of hydrated CO_2 is 6.37, the concentration of CO_2 at pH 5.8 is approximately 4 fold the concentration at pH 6.8.) If bicarbonate was the active form, the same concentration should show a larger effect at pH 6.8 than at 5.8. Our results indicate that bicarbonate ions are very probably the active species.

The data presented in Table 1 show that at all CO_2/HCO_3^- concerntrations below 20 mM, much larger stimulation in DCPIP reduction is observed at pH 6.8 than pH 5.8. For example, 0.5 mM $NaHCO_3$ increases the rate of Hill reaction 2.4 fold at pH 6.8 but only 1.3 fold at pH 5.8. At pH 6.8, 5 mM $NaHCO_3$ is nearly a saturating concentration producing a 4.3 fold increase while this concentration, at pH 5.8, is still far below saturation, producing a 2.2 fold increase.

Upon examining these data, (Table 1) one also notices a fairly abrupt 2 fold increase in the rate of DCPIP reduction in going from 5 to 20 mM $NaHCO_3^-$ at pH 5.8. We do not know the reason for this jump, and hesitate to speculate extensively without establishing its true importance.

Table 1

Initial Rate of DCPIP Reduction as a Function of CO_2/HCO_3^- Concentration at pH 5.8 and 6.8

pH	$NaHCO_3$	Rate	$+HCO_3^-/-HCO_3^-$
	mM	μmoles DCPIP reduced mg^{-1} Chl hr^{-1}	Ratio
5.8	0	17.3 ± 3.6	--
	0.1	17.6 ± 4.8	1
	0.5	23.3 ± 3.4	1.3
	1.0	24.4 ± 3.0	1.4
	5.0	38.2 ± 3.9	2.2
	20.0	78.8 ± 9.3	4.5
6.8	0	15.2 ± 2.6	--
	0.1	23.3 ± 5.1	1.5
	0.5	43.8 ± 6.5	2.4
	1.0	47.0 ± 4.9	3.1
	5.0	65.7 ± 2.7	4.3
	20.0	71.6 + 5.1	4.7

The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer, 39 μM DCPIP and 15 μg chl/ml of chloroplast suspension. Saturating red light was 2 x 10^6 ergs cm^{-2} sec^{-1}. The date are the average of 5 experiments.

Similar results were observed when ferricyanide was used as a Hill oxidant. Ferricyanide reduction was in this case measured by the method of Avron and Shavit (33). Again, low concentrations of bicarbonate stimulated Hill activity more at pH 6.8 than at pH 5.8.

One may still argue that CO_2 is the active species by proposing that at the lower pH, the affinity of the active site for CO_2 is lowered so that even though CO_2 occurs at higher concentration at pH 5.8, it has less stimulating effect. We feel this is less likely, since it requires this added assumption. Moreover, it is difficult to imagine how uncharged CO_2 can possess the binding qualities required to explain other data presented later (results and discussion of Table 4).

Finally, Professor Helmut Metzner (personal communication) obtained additional evidence that HCO_3^- is the active species producing the effect. In his experiments, addition of HCO_3^- to his reaction mixture produced an immediate stimulation of O_2 evolution whereas addition of CO_2 gas required a noticable lag period before O_2 evolution was affected.

Stimulation of the Hill reaction by HCO_3^- rather than CO_2 may explain the requirement of high salt concentration for maximum bicarbonate effect (7,10). Since bicarbonate ion, not CO_2 gas, is very probably the active species, it is reasonable to suggest that anions (chloride and particularly acetate) compete with bicarbonate for binding sites on the chloroplast. The higher the concentration of the ions, the more effective they will be at removing bicarbonate and keeping it from the active sites. Since the need for bicarbonate is very specific (8) such competitive action by other ions can effectively reduce Hill activity. It is also of interest that once bicarbonate is removed from chloroplasts, bicarbonate dependence remains even after competing anions are removed. Other experiments (data not presented here) indicate that high salt concentrations are present during the bicarbonate depletion procedure, prior to assay, large bicarbonate stimulation can be observed in reaction mixture containing no acetate and only small amounts of NaCl. High salt concentration was left in the reaction mixture routinely only to minimize the effect of contamination of solutions with atmospheric CO_2.

B. Bicarbonate Stimulation of Hill Activity Using Glutaraldehyde Fixed Chloroplasts

We examined the possibility that HCO_3^- stimulates the Hill reaction by causing some sort of gross conformational change in the chloroplast membrane. It was reasoned that chloroplasts in which such changes were blocked by glutaraldehyde fixation would not demonstrate a bicarbonate effect. The effect was, however, present.

The results presented in Table 2 show that while glutaraldehyde treatment of chloroplasts reduces the stimulation of DCPIP reduction bicarbonate as compared to the normal (2.75 fold increase versus 4.78 for the normal), a large effect is still observed. The reduced bicarbonate effect may be attributed to non-specific causes since glutaraldehyde treatment also reduces the overall activity (34).

Similar results were obtained when ferricyanide was the Hill oxidant.

C. Bicarbonate Effect on an Artificial Electron Donor System

The following experiment was done to locate the site of action of bicarbonate along the electron transport chain. Diphenylcarbazide

Table 2

Initial Rate of DCPIP Reduction Using Normal and Glutaraldehyde-fixed Chloroplasts in the Presence and the Absence of Bicarbonate

Chloroplasts	Rate $-NaHCO_3$	 +0.02 M $NaHCO_3$	$+HCO_3^-/-HCO_3^-$
	μmoles DCPIP reduced mg^{-1} Chl hr^{-1}		Ratio
Normal	16.0 ± 1.3	76.5 ± 6.6	4.78
Glutaraldehyde-fixed	8.25 ± 1.6	22.7 ± 2.4	2.75

Reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer pH 6.8, 30 μM DCPIP, and 20 μg chl/ml of chloroplast suspension. Saturating red light was 2 X 10^6 ergs cm^{-2} sec^{-1}. Initial condition was anaerobic. The data are the average of 5 experiments. Chloroplasts were fixed prior to depleting them of HCO_3^- in 2 percent glutaraldehyde.

(DPC), an artificial electron donor to photosystem II (13), was given to chloroplasts after heat treatment which destroyed their ability to evolve oxygen. It was expected that if bicarbonate acted between the oxygen evolving site and the site of electron donation by DPC, no effect of bicarbonate would be observed on the rate of electron flow from DPC to DCPIP. This reaction was found to be insensitive to bicarbonate ions (Table 3). Thus, at least one site of action of bicarbonate is before the site of donation of electrons by DPC, *i.e.*, on the oxygen evolving side of the photosystem II.

Table 3 indicates that while normal chloroplasts show a large bicarbonate effect (4.4 fold increase with bicarbonate) when DCPIP reduction is coupled to the natural electron donor, only a very small effect is seen when DPC is the electron donor (1.12 fold increase with bicarbonate). Heated chloroplasts, without DPC, also show a trace more DCPIP reduction with bicarbonate. DCMU (10 μM) prevented DCPIP reduction when DPC donated electrons, as expected. The very slight stimulation of bicarbonate on the DPC/DCPIP system, if it is real, may be due to the stimulation of a trace amount of normal electron flow parallel to the artificial flow as when DPC is ommitted from the reaction mixture (line 3).

Thus if we assume that there is an electron transport system between Z (electron donor to reaction center II chlorophyll) and the oxygen evolving site, bicarbonate must act on that system. If, on the other hand, we assume that Z itself is part of the oxygen evolving site, then we might postulate that, while DPC can donate electrons to the first oxidation state of the reaction center *i.e.* Z^+, bicarbonate is somehow necessary for the accumulation of the four positive charges thought necessary for oxygen evolution, according to the present

Table 3

Initial Rate of DCPIP Reduction in Normal and Heat Treated Chloroplasts With DPC as Electron Donor With and Without Added Bicarbonate

Treatment	Rate $-NaHCO_3$	$+0.01$ M $NaHCO_3$	$+HCO_3^-/-HCO_3^-$
	μmoles DCPIP reduced mg^{-1} Chl hr^{-1}		Ratio
(1) None	15.8 ± 3.8	70.0 ± 6.2	4.4
(2) Heat	0.00	trace	--
(3) Heat + 0.05 mM DPC	50.0 ± 8.9	56.0 ± 11.6	1.12
(4) Heat + 0.05 mM DPC + 50 μM DCMU	0.0	0.0	--

Reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate 0.05 M phosphate buffer pH 6.8, 39 μM DCPIP, and 15 μg chl/ml of chloroplast suspension. Saturating red light was 2 X 10^6 ergs cm^{-2} sec^{-1}. The data are the average of 5 experiments.

model of Kok, Forbush and McGloin (35). We now have some data to support this hypothesis [Stemler _et al_. (36)]. In either case bicarbonate must act very close to the oxygen evolving site.

D. The Effect of Dark Incubation and Light Pretreatment at Various Bicarbonate Concentrations on Hill Activity

Table 4 (lines 1 and 2) show that if normal chloroplasts, _i.e._, not HCO_3^- depleted, are incubated for 5 minutes in 0.25 M NaCl and 40 mM Na acetate in the absence of bicarbonate, they slowly lose DCPIP reducing activity. Five minutes of incubation in the presence of 20 mM Na HCO_3 as well, produces no loss in activity. This confirms the findings of Good (8) and West and Hill (11). However, if one begins with bicarbonate depleted chloroplasts, dark incubation in the presence of bicarbonate can restore DCPIP reducing activity. Comparing lines 3 and 4, it is apparent that, depending on bicarbonate concentration, a 5 minute dark incubation period between suspending the chloroplasts in reaction mixture and assay can markedly increase the rate of reaction. In the absence of bicarbonate, and again at saturating concentrations (20 mM) a 5 minute dark period has little effect in increasing activity. An incubation period of 5 minutes in 1 mM $NaHCO_3$, on the other hand, increases the initial rate of DCPIP reduction and, in 0.1 mM bicarbonate, the rate is doubled. In fact, incubating chloroplasts in the dark for 5 minutes at 0.1 mM $NaHCO_3$ (line 4) produces the same initial rate of Hill reaction as 1 mM without dark incubation (line 3).

Comparing lines 3 and 6, if instead of a dark period, Hill reaction is allowed to proceed in saturating light, no increase is seen in the rate of reaction after two minutes regardless of the bicarbonate

Table 4

Initial Rate of DCPIP Reduction With Normal and Bicarbonate-Depleted Chloroplasts As A Function Of Bicarbonate Concentration and Various Light and Dark Pretreatments

Chloroplasts	Incubation time and pretreatment	NaHCO$_3$ concentration during assay 0.0	 0.1mM	 1mM	 20mM
			μmoles DCPIP reduced mg^{-1} Chl hr^{-1}		
Normal	(1) None	91.2±4.7	--	--	85.5±7.8
	(2) 5 min dark	71.2±7.8	--	--	82.0±6.9
HCO$_3^-$ depleted	(3) none	15.2±2.6	23.3±5.1	47.0±4.9	71.6±5.1
	(4) 5 min dark, + DCPIP	13.3±3.7	48.5±4.6	66.5±8.3	75.0±8.9
	(5) 5 min dark, - DCPIP	--	47.3±7.7	--	--
	(6) 2 min saturating light, + DCPIP	14.1±2.2	24.7±1.9	42.0±1.6	59.4±4.3
	(7) 2 min saturating light, + DCPIP, + 5 min dark	18.3±2.4	40.6±2.7	64.3±3.5	54.5±6.7
	(8) 2 min saturating light, - DCPIP, + 5 min dark	--	--	--	72.5±6.3

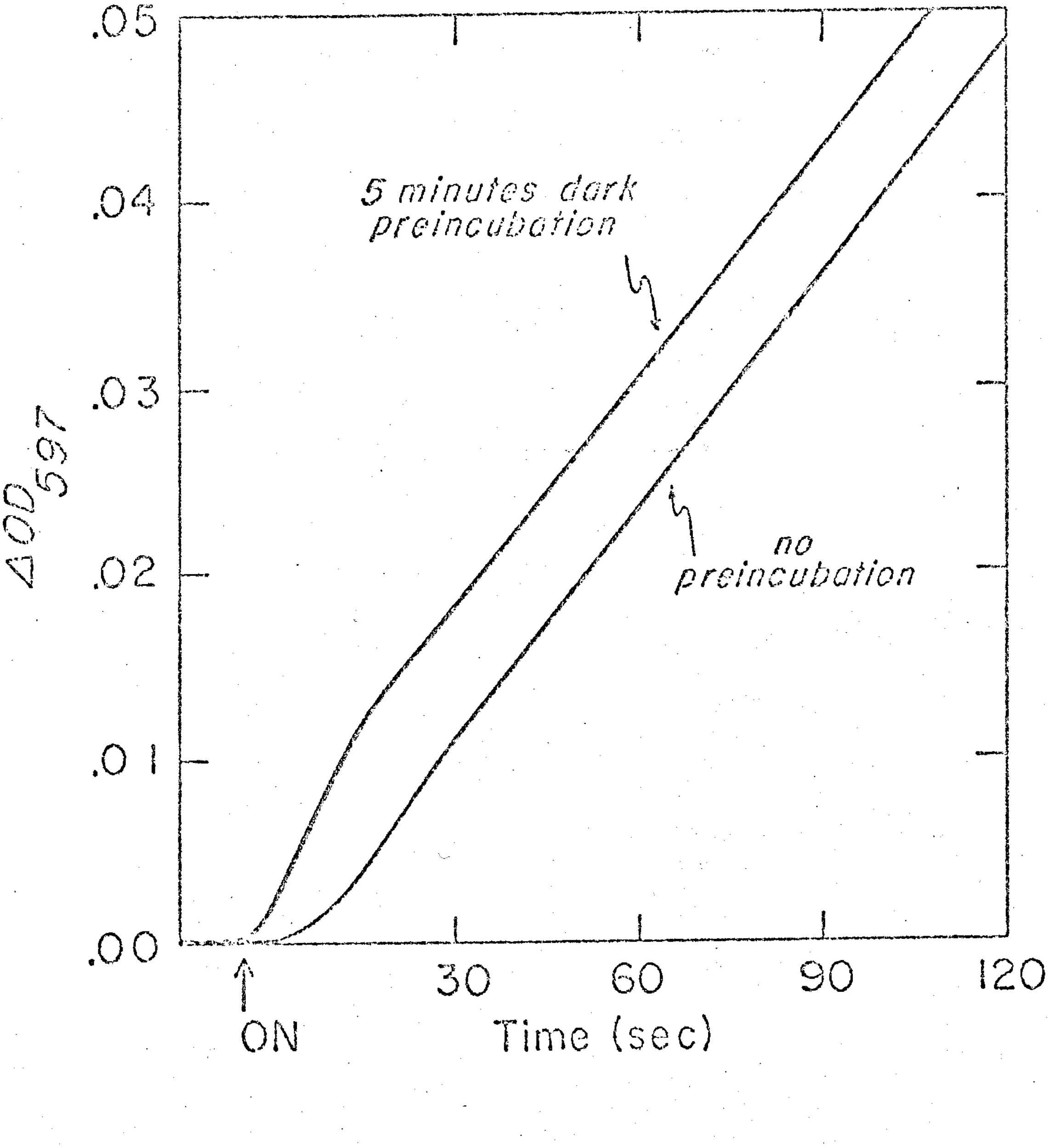
Reaction conditions were the same as in Table 3. Chloroplasts were suspended in the reaction mixture containing 39 μm DCPIP for pretreatment except where indicated (lines 5 and 8) in which case the dye was injected immediately after pretreatment. The data are the average of 5 experiments.

concentration. At high bicarbonate concentration (20 mM) an irreversible decrease is seen (a symptom of photoinactivation which is generally accelerated by increased electron flow; compare line 6 with line 8). Finally, if a 5 minute dark period is given subsequent to 2 minutes of active Hill reaction, an increase in the initial rate is again observed at 0.1 mM and 1.0 mM bicarbonate (line 7).

Differences in the initial rate of DCPIP reduction with and without 5 minutes dark incubation are evident in the recorder traces presented in Figure 7. The bicarbonate concentration was 0.1 mM. Without dark pretreatment, the rate of reduction, after a short lag of a few seconds, is nearly linear for the first few minutes. With a 5 minute dark preincubation, a high rate of DCPIP reduction upon illumination is first observed until a change in slope is seen between 10 and 20 seconds. From that point onwards, the two curves run parallel indicating nearly identical rates of reaction. The increased activity due to preincubation in the dark therefore is seen to be temporary. The curve for the 5 minute dark incubated chloroplasts (Figure 7) was selected to show the most rapid change in slope. Usually the change is more gradual, occurring over a number of seconds.

To explain these results we again propose that bicarbonate binds to active sites on the chloroplast in the dark. Moreover it appears that bicarbonate may be released again from the chloroplast as the Hill reaction proceeds. We do not observe an increased rate with time if chloroplasts are suspended in bicarbonate and the Hill reaction is actually taking place (Table 4). The increased rate develops only in the dark. This suggests that while bicarbonate may be binding to the chloroplast even during the Hill reaction, it is released again as

Figure 7. Rate of DCPIP reduction by maize chloroplasts previously depleted of bicarbonate, with and without 5 minutes dark preincubation in 0.1 mM $NaHCO_3$. Other reaction conditions were the same as in Table 3. (ΔOD_{597} = absorbance change at 597 nm.)


.05
.04
.03
.02
.01
.00
ΔOD_{597}
5 minutes dark preincubation
no preincubation
30
60
90
120
ON
Time (sec)

rapidly as it binds. The kinetic differences in the rate of DCPIP reduction seen in Figure 7 can also be explained in terms of a release of bicarbonate from the chloroplast. We propose that a reserve of (bound) bicarbonate is built up during 5 minutes dark incubation in chloroplasts suspended in 0.1 mM bicarbonate. This reserve accounts for the increased rate of reaction upon illumination. The change in slope occurs when this reserve is dissipated i.e., bicarbonate is released. After the "excess" bicarbonate is released, the reaction rate is again dependent on the dark binding rate of bicarbonate. The proposal that bicarbonate is released during Hill reaction is further supported by the preillumination studies already discussed in Chapter III.

West and Hill (11) pointed out that the bicarbonate concentration required to achieve saturation in their experiments differed from other workers, but offered no explanation. We now see that the effect of a given concentration of bicarbonate depends not only on the pH and the concentration of competing anions, but also on the time between suspending the chloroplasts in bicarbonate solution and assay. This time interval required for rebinding of bicarbonate is especially noticeable at lower concentrations and may very well have differed among investigators. The assumption of strong ionic binding characteristics of bicarbonate may also explain why certain investigators have seen little or no effect of exogenous bicarbonate on the Hill reaction (6). The bicarbonate already present must first be removed. In this regard it is questionable whether or not CO_2 can be removed from alkaline solutions. The production of carbonate free standard base solutions is not a simple matter (37). If most of the CO_2 is in the bicarbonate or carbonate ionic form, as is increasingly the case as one exceeds pH 7.0, it will not be removed by boiling or bubbling with another gas. Moreover the presence of any organic matter to which these ions can bind may very well cause complications, making CO_2 removal even more difficult at high pH. Thus a failure to see an effect of exogenous bicarbonate at pH 8.0 (38), in contrast to a lower pH, can mean either that bicarbonate plays no role at the pH or, more likely, it was not adequately removed from the control.

V. THE EFFECTS OF BICARBONATE ION ON CHLOROPHYLL A FLUORESCENCE TRANSIENTS AND DELAYED LIGHT EMISSION

A. Chlorophyll a Fluorescence Transients

1. High Light Intensity

Figure 8 shows the fast fluorescence transient observed in isolated chloroplasts exposed to 8 x 10^4 ergs cm^{-2} sec^{-1} blue-green light. These traces were obtained with an Esterline Angus recorder. Careful and repeated measurements, using the faster oscillographic recorder, showed no effect of bicarbonate ions on the "O" level (i.e., the constant fluorescence; Fo). However, in bicarbonate depleted chloroplasts the initial fluorescence rise from 0 to I (intermediate hump) fluorescence is rapid but the I → D (the dip) and the D → P (peak; F_∞) phases are slow. In the presence of 10 mM bicarbonate, the 0 → I rise is slower and depressed, the dip D is not clear and occurs earlier (see insert Figure 8), and the D → P rise is much more rapid than in bicarbonated-depleted chloroplasts. The intensity of fluorescence at the P level, like the 0 level, is insensitive to HCO_3^-. In saturating light (as used here), when 3- (3, 4-dichlorophenyl)-1, 1 dimethylurea (DCMU) is present, the fluorescence transient is not noticeably different with and without HCO_3^-. The rise of P is very rapid, as expected, compared to the unpoisoned chloroplasts, but the extent of the P level is lower in both cases.

If unpoisoned chloroplasts are subjected to 8 x 10^4 erg cm^{-2} sec^{-1} light until the P level is achieved, recovery of the fluorescence transient is incomplete, regardless of the dark time allowed (up to 10 min.; see Figure 9). Recovery is, however, faster and more complete in the

Figure 8. Fluorescence yield of chlorophyll a at 685 nm as a function of time of high intensity illumination in the presence and absence of 0.01 M $NaHCO_3$. Maize chloroplasts previously depleted of HCO_3^- were suspended in 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer pH 6.8, ± 10 μm DCMU; 10 μg chlorophyll/ml. Blue actinic light, 8 x 10^4 ergs cm^{-2} sec^{-1}.

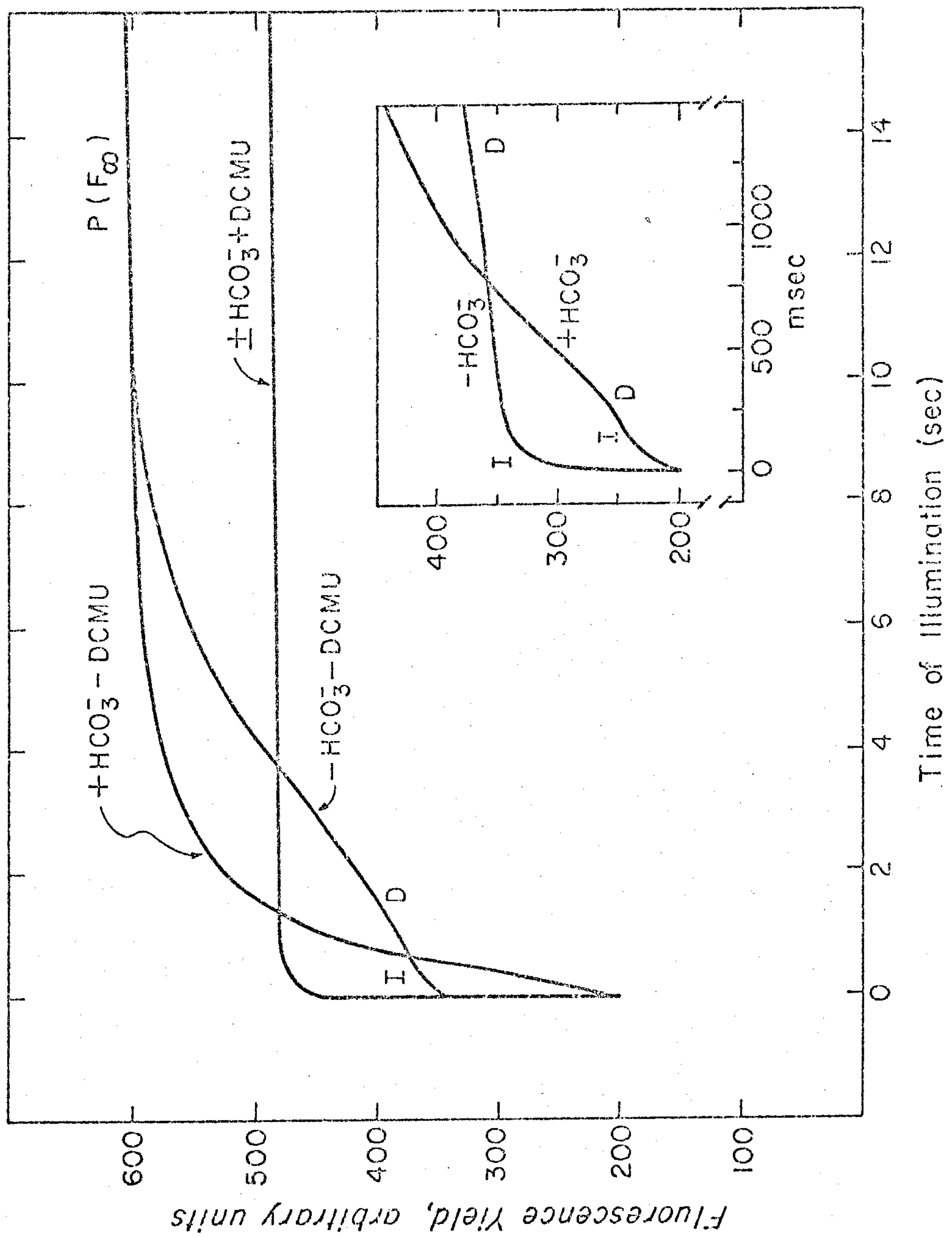
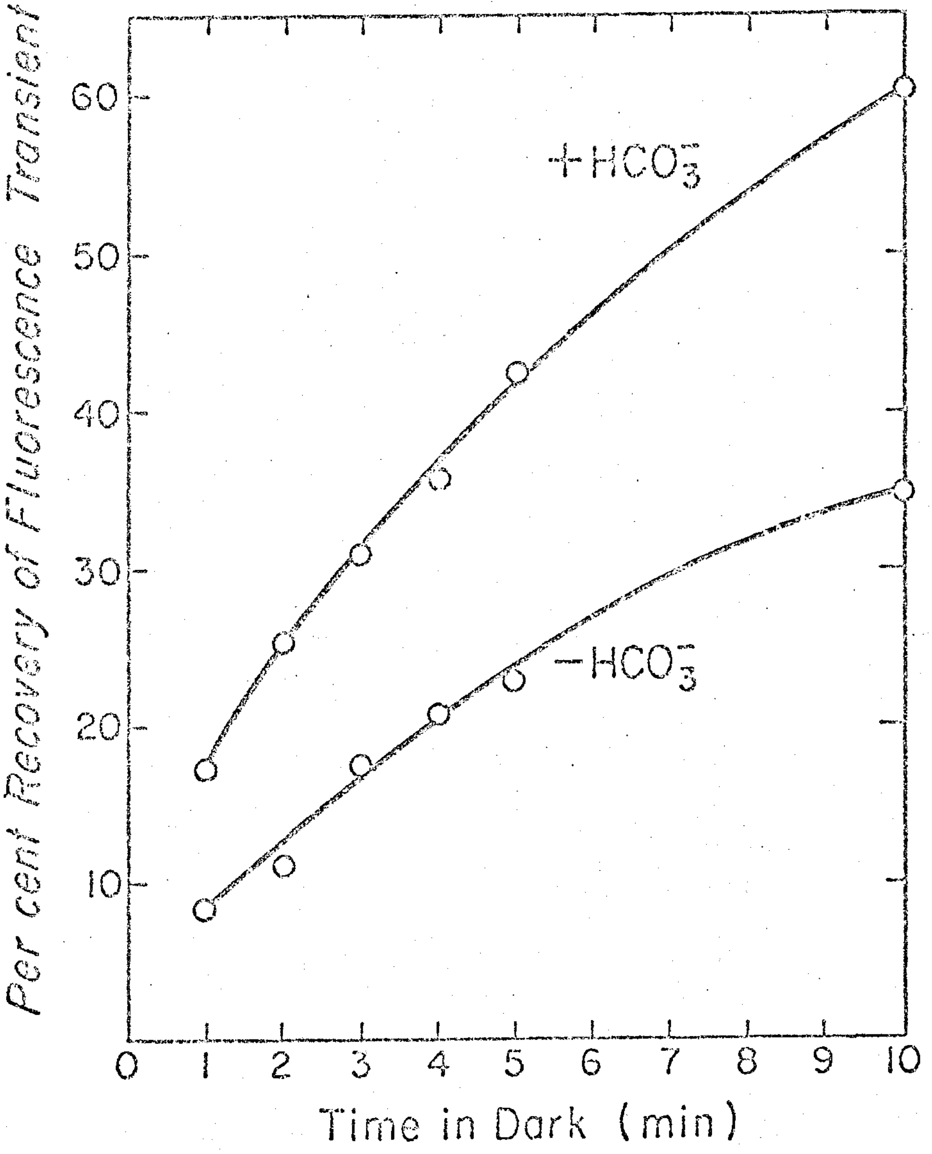

P (F∞)
±HCO3⁻+DCMU
+HCO3⁻−DCMU
−HCO3⁻−DCMU
I
D
600
500
400
300
200
100
0
2
4
6
8
10
12
14
Time of Illumination (sec)
Fluorescence Yield, arbitrary units
400
300
200
0
500
1000
msec
−HCO3⁻
+HCO3⁻
I
D
I
D

Figure 9. Recovery of the chlorophyll a fluorescence transient in the dark using unpoisoned chloroplasts. Conditions were the same as in Figure 8.


Per cent Recovery of Fluorescence Transient
Time in Dark (min)
+HCO$_3^-$
−HCO$_3^-$
10
20
30
40
50
60
0
1
2
3
4
5
6
7
8
9
10

presence of HCO_3^-. After one minute of darkness, about 8.5 percent of the transient is recovered in HCO_3^- depleted chloroplasts while about 17 percent is recovered in chloroplasts given 10 mM HCO_3^-; after 10 minutes dark, values are 35 and 60 percent respectively.

2. Low Light Intensity

Fluorescence transients were also measured at low light intensity (400 ergs cm^{-2} sec^{-1}) in order to allow comparison with DLE results to be reported here. At this intensity, fluorescence is much greater in the presence of DCMU (see Figure 10). HCO_3^- slows the rise to steady state, and lessens the extent of rise. In the absence of DCMU, very little variable fluorescence is observed although a slightly greater amount is seen in the absence of HCO_3^-.

The recovery of low intensity fluorescence transients was measured, using DCMU poisoned chloroplasts, with dark times up to 16 seconds only (Figure 11). In the presence of HCO_3^-, about 14 percent of the transient is recovered within 2 seconds. Very little additional recovery is seen in the following 14 seconds. In the absence of HCO_3^-, only 2-3 percent of the transient is recovered with in 2 seconds. Recovery increases to about 8 percent at 6 seconds and increases slowly to 10 percent at 16 seconds. Even after long periods of dark, recovery of the transient remains far from complete whether or not HCO_3^- is present. After 2 minutes (data not shown), only about 35 percent of the transient is recovered and this is the same in the presence and absence of HCO_3^-. It must be pointed out that a higher steady state (P) level was used in calculating recovery (for Figure 11) in the absence of bicarbonate than in its presence (see Figure 10). This is significant because we

Figure 10. Fluorescence yield of chlorophyll a at 685 nm as a function of time of low intensity illumination. Blue actinic light, 400 ergs cm^{-2} sec^{-1}. Other conditions were the same as in Figure 8.

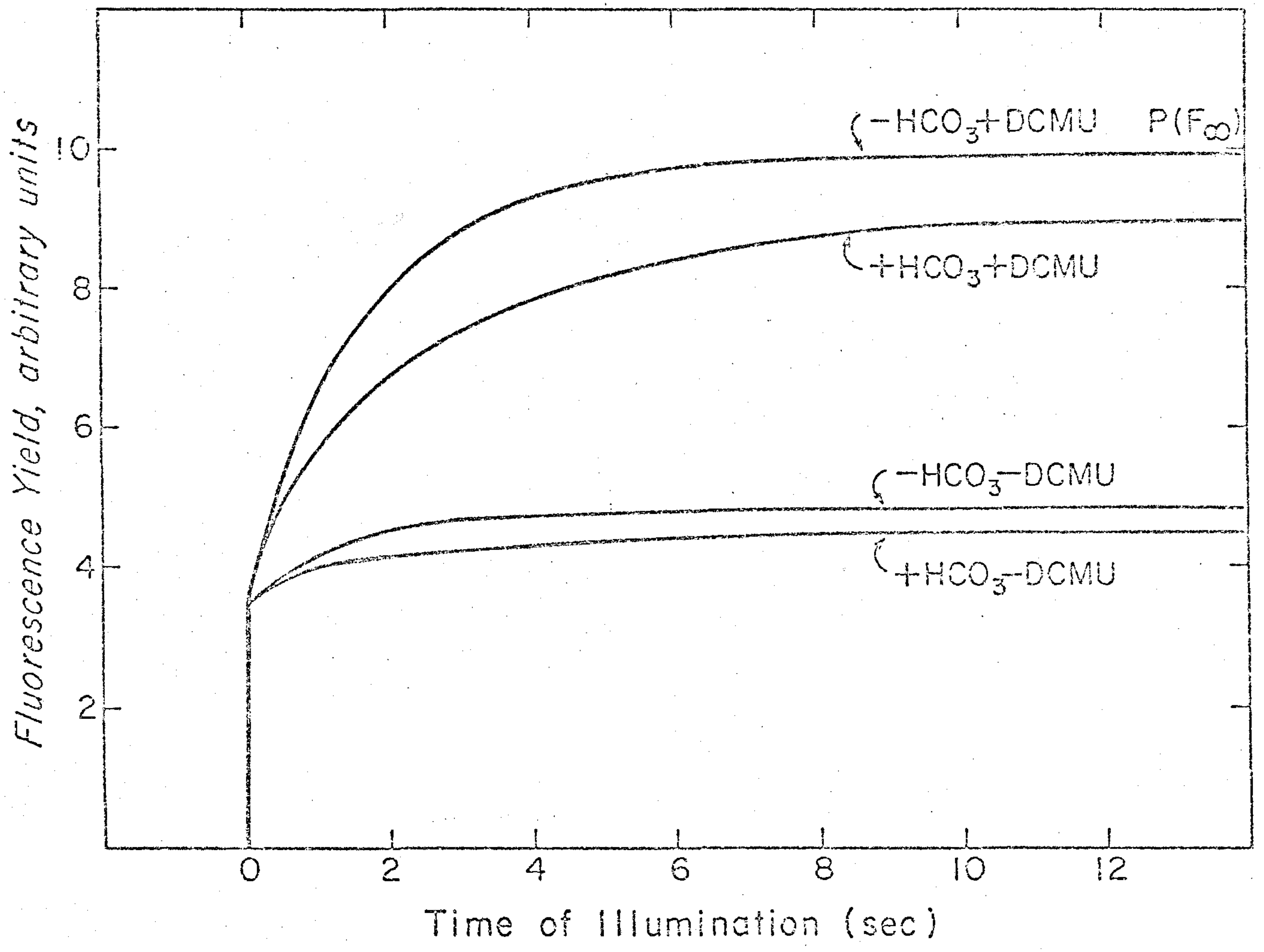
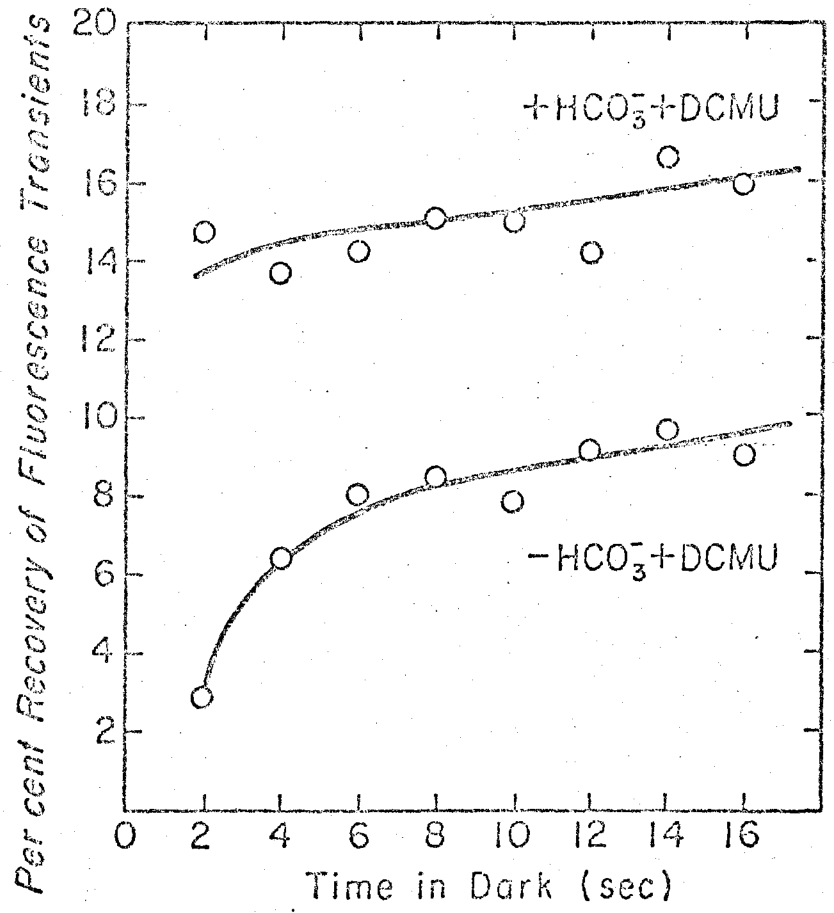

$-HCO_3+DCMU$
$P(F_\infty)$
$+HCO_3+DCMU$
$-HCO_3-DCMU$
$+HCO_3-DCMU$
Fluorescence Yield, arbitrary units
10
8
6
4
2
0
2
4
6
8
10
12
Time of Illumination (sec)

Figure 11. Recovery of the chlorophyll a fluorescence transient in the dark using DCMU poisoned chloroplasts after illumination with weak blue light, 400 ergs cm^{-2} sec^{-1}. Other conditions were the same as in Figure 8.


Per cent Recovery of Fluorescence Transients
20
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
Time in Dark (sec)
$+HCO_3^- + DCMU$
$-HCO_3^- + DCMU$

propose (see later) that the lower P level in the presence of HCO_3^- is due to a rapid primary back reaction, i.e., charge recombination, taking place. Consequently, more photosystem II traps stay open during illumination in the presence of HCO_3^- and variable fluorescence is suppressed. Since recovery was calculated on the basis of what occurred in the dark, only the percent of photosynthetic units actually closed at the termination of light are considered in the recovery data. In other words, the percent of traps capable of very fast recovery in the presence of HCO_3^- is probably much higher than the 14 percent indicated in Figure 11.

B. Delayed Light Emission

Figure 12 shows the delayed light emitted, by chloroplasts, seconds after illumination for 10 seconds with 400 ergs cm^{-2} sec^{-1} blue light. The greatest amount of delayed light is observed when chloroplasts are illuminated in the presence of both HCO_3^- and DCMU. When DCMU is absent, the intensity of DLE is reduced by slightly more than 50%. Still less (about 25% of the maximum) is observed when both HCO_3^- and DCMU are omitted. However, the least DLE (about 10% of the maximum) is seen when HCO_3^- is omitted but DCMU is present. Thus, the presence of 0.01M HCO_3^- increases DLE whether DCMU is present or absent.

When chloroplasts are illuminated for 60 seconds at low intensity (400 ergs cm^{-2} sec^{-1}), the pattern of DLE is different (Figure 13). The amount of DLE is greatest in the presence of HCO_3^- but in the absence of DCMU. A large increase (about 3 fold) is seen compared to the 10 second value. Relatively less (about 70% of the maximum) DLE is observed when HCO_3^- and DCMU were both present as this amount of DLE

Figure 12. DLE (delayed light emission) decay from maize chloroplasts previously depleted of HCO_3^- illuminated for 10 sec in weak blue light (400 ergs cm^{-2} sec^{-1}). Chloroplasts were suspended in 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer pH 6.8, ± 0.01 M $NaHCO_3$ ± 10 μ M DCMU. 15 μg chlorophyll -1 ml suspension.

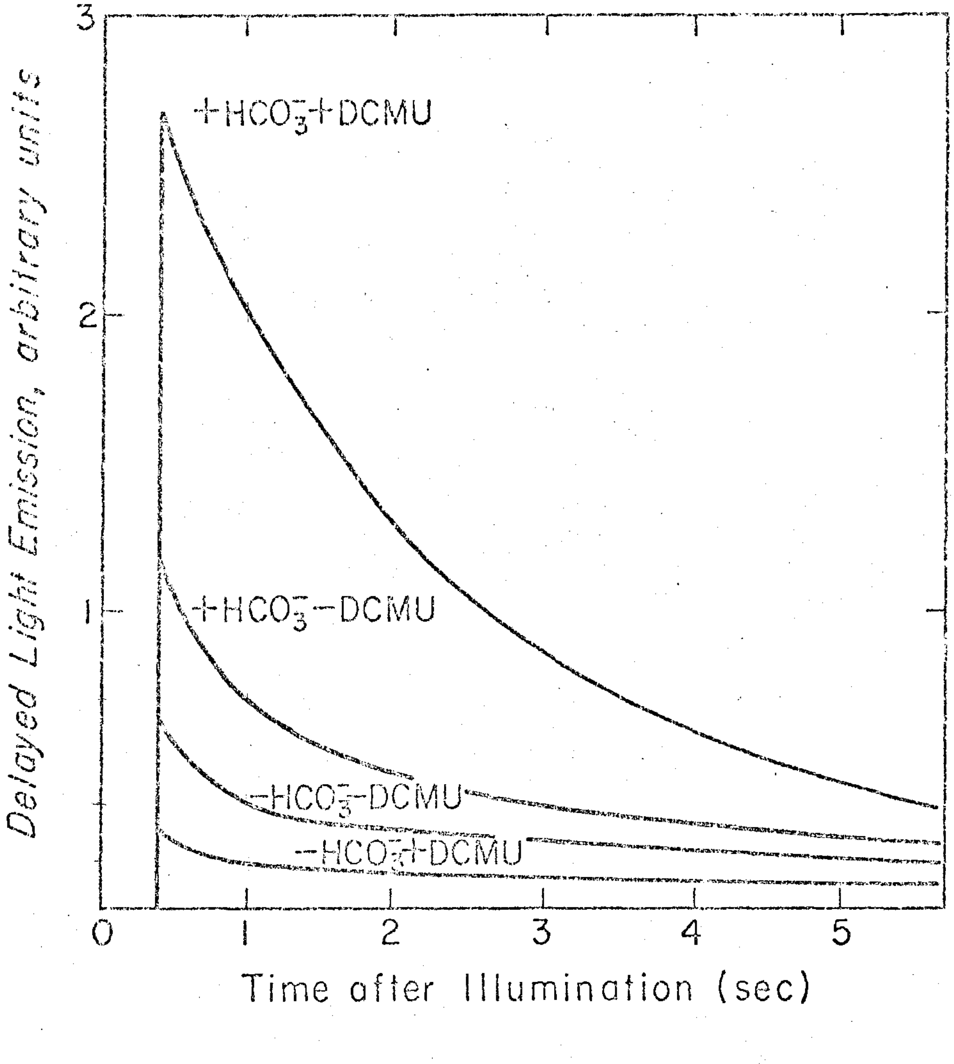
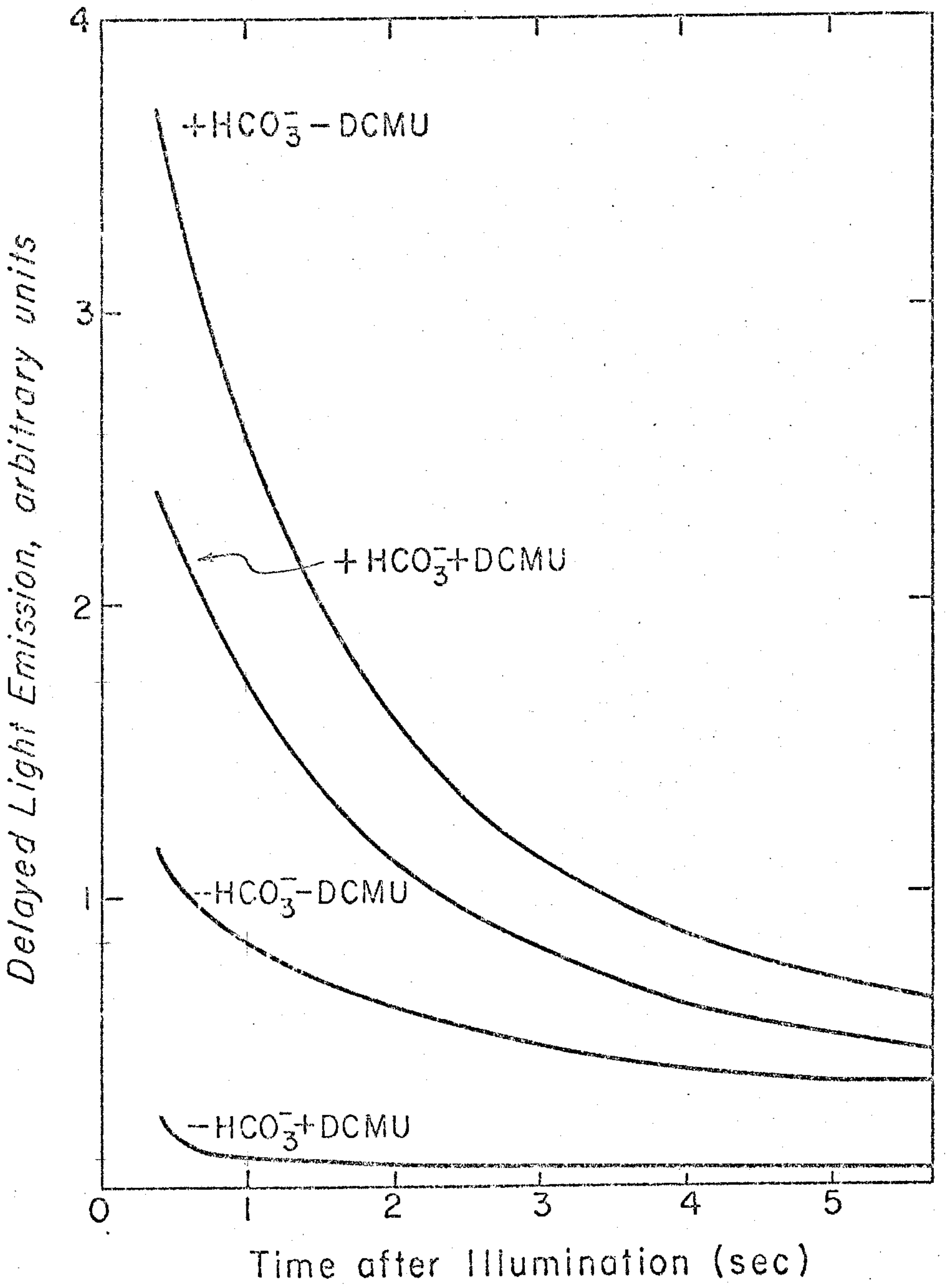

Delayed Light Emission, arbitrary units
Time after Illumination (sec)
3
2
1
0
1
2
3
4
5
+HCO$_3^-$+DCMU
+HCO$_3^-$−DCMU
−HCO$_3^-$−DCMU
−HCO$_3^-$+DCMU

Figure 13. DLE decay from maize chloroplasts illuminated for 60 secs. All other conditions exactly as described in the legend of Figure 12.


4
3
2
1
0
1
2
3
4
5
Delayed Light Emission, arbitrary units
Time after Illumination (sec)
$+HCO_3^- - DCMU$
$+HCO_3^- + DCMU$
$-HCO_3^- - DCMU$
$-HCO_3^- + DCMU$

remained essentially unchanged from the 10 second value. The amount of DLE seen in the absence of both HCO_3^- and DCMU increased (about 2 fold) from the 10 second value but remained less (about half as much) than observed in the presence of both treatments. The least amount of DLE (about 5% of maximum) is observed in the absence of HCO_3^- but in the presence of DCMU; here an actual decline from the 10 second value occurs. Again, the presence of HCO_3^- increases DLE whether DCMU is present or absent.

Short periods (10 seconds) of higher illumination (8×10^3 ergs cm^{-2} sec^{-1}; data not shown) produced results very similar to longer periods (60 seconds) of weak light (Figure 13).

C. Delayed Light Emission Induced by DCMU Injection

If DCMU is injected into a chloroplast suspension as DLE is being monitored after a 10 second illumination with weak blue light, a large increase in light emission results (Figure 14). This effect was first observed by Clayton (39) and confirmed by Lavorel (40). We see, however, that HCO_3^- must be present during illumination to observe this effect. Our new finding is that bicarbonate-depleted chloroplasts show no DLE jump upon injection of DCMU. After 60 seconds of the same illumination, a slightly different effect is seen (Figure 15). Chloroplasts illuminated in the presence of HCO_3^- showed a less dramatic DLE jump, and HCO_3^--depleted chloroplasts demonstrated an actual drop in DLE upon injection with DCMU.

The opposite experiment, injecting HCO_3^- while monitoring DLE from HCO_3^--depleted chloroplasts, was also done. No effect on DLE was observed upon injection of HCO_3^- whether or not the chloroplasts were

Figure 14. DLE decay from maize chloroplasts previously depleted of HCO_3^- illuminated for 10 seconds in weak blue light (400 ergs cm^{-2} sec^{-1}) 0.2 ml DCMU injected where indicated, final concentrations, 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer pH 6.8, ± 0.01 M $NaHCO_3$, 15 μg chlorophyll ml^{-1} suspension.

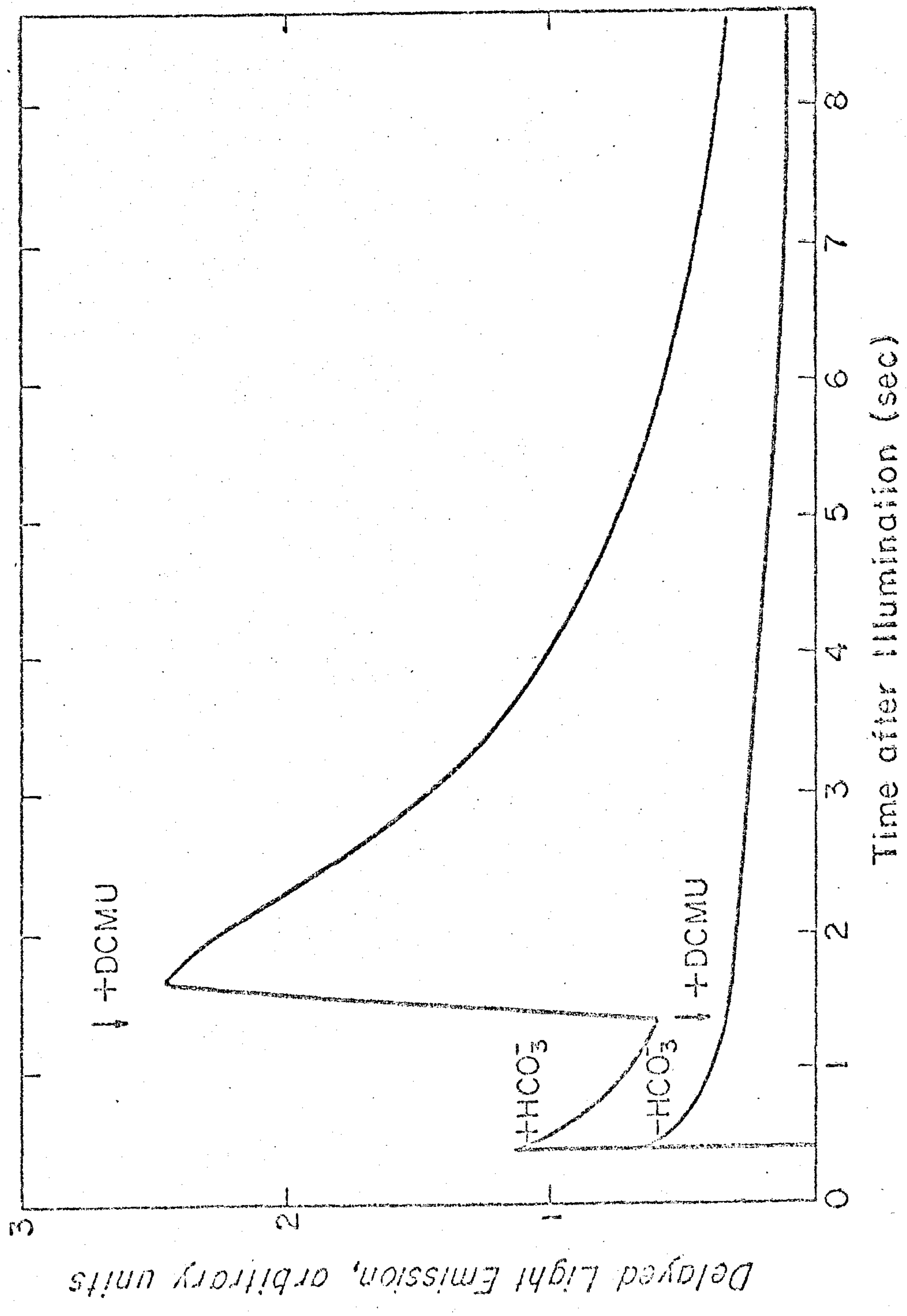
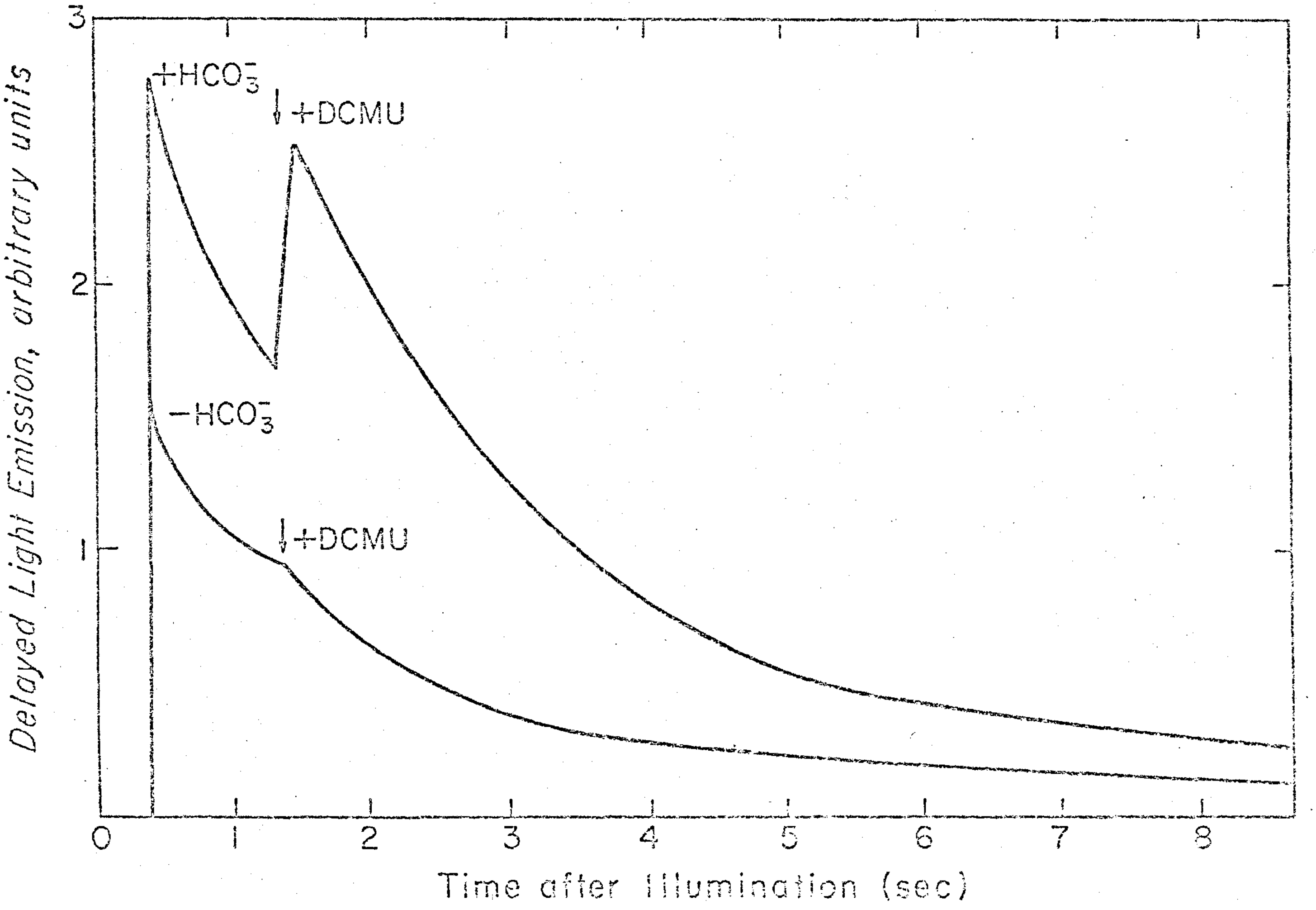

+DCMU
+HCO$_3^-$
−HCO$_3^-$
+DCMU
Delayed Light Emission, arbitrary units
0
1
2
3
Time after Illumination (sec)
0
1
2
3
4
5
6
7
8

Figure 15. DLE decay from maize chloroplasts illuminated for 60 seconds. All other conditions exactly as described in the legend of Figure 14.



3
2
1
0
+HCO$_3^-$
+DCMU
−HCO$_3^-$
+DCMU
Delayed Light Emission, arbitrary units
0
1
2
3
4
5
6
7
8
Time after Illumination (sec)

poisoned with DCMU during illumination (data not shown). Short (10 second) periods of illumination subsequent to HCO_3^- injection did yield an increased DLE, however. It appears, therefore, that HCO_3^-, unlike DCMU, must be present during illumination to affect DLE.

D. A Working Hypothesis

To attempt to understand these data, we draw attention first to the simpler DCMU inhibited system, thus avoiding the need for reference to electron flow beyond the electron acceptor Q of photosystem II. After long periods of darkness photosystem II reaction centers, according to Kok and coworkers (see Forbush <u>et al</u>., 41; Mar and Govindjee, 42), are in, or decay to, the oxidation states S_o or S_1 meaning that the electron donor Z is either electrically neutral or has a single positive charge. Illuminating such a system in the presence of DCMU, therefore, one expects to observe the effect of only the following reaction:

$$S_o \xrightarrow{h\nu} S_1;\ (\mathrm{ZChl\ Q} \xrightarrow{h\nu} \mathrm{Z^+Chl\ Q^-}\ //) \qquad (1)$$

$$S_1 \xrightarrow{h\nu} S_2;\ (\mathrm{Z^+Chl\ Q} \xrightarrow{h\nu} \mathrm{Z^{2+}Chl\ \ Q^-}//) \qquad (2)$$

where Q is the electron acceptor.

Which of these reactions predominates, of course depends on the initial S_o/S_1 ratio. It is believed that the S_o and S_1 states are stable for long periods in the dark, although this may be true only in the normal unpoisoned chloroplast system. [It is now known that the S_o/S_1 ratio can be changed chemically in the dark (Bouges-Bouquet, 43.]

The S_2 state (Z^{2+}), in contrast, decays relatively quickly (Joliot et al., 44) yielding relatively large amounts of delayed light (Barbieri et al., 45). (Also see Lavorel, 40.)

Based on the above model, we propose, as one working hypothesis, that the HCO_3^- ion may be complexing with the photosystem II reaction centers either forming, or somehow stabilizing a population of reaction centers in the S_1 state and allowing transition for these centers to higher oxidation states. (In the presence of DCMU, however, higher states would be restricted to S_2.)

We may now explain the Chl a fluorescence transients seen using low intensity actinic light (Figure 10). The rise of the fluorescence is slower in the presence of HCO_3^- than in its absence. Since the initial charge separation is completely photochemical, the forward reduction of Q is assumed to take place at the same rate whether or not HCO_3^- is present. A delay in the fluorescence rise is, therefore, attributed to a rapid primary back reaction (charge recombination) in the presence of HCO_3^-. This, in turn, is expected if a significant number of reaction centers are making the transition $S_1 \xrightarrow{h\nu} S_2$, the S_2 state being unstable. In the presence of HCO_3^-, rapid partial recovery of the fluorescence transient in the dark (Figure 11) and higher DLE when the exciting light is terminated (Figure 12) support the above suggestion. If, in contrast, HCO_3^--depleted chloroplasts undergo to a greater extent the (relatively) irreversible $S_o \xrightarrow{h\nu} S_1$ transition, we expect a more rapid rise in variable fluorescence (Figure 10) a slower and more incomplete recovery (Figure 11), and lower intensity of delayed light (Figure 12).

In examining further the data obtained with DCMU poisoned systems, an interesting complication becomes apparent. DLE "ceases" for the most part by about 10 seconds after termination of the exciting beam, yet the recovery of the fluorescence transient is far from complete at this time in our samples. This implies that a large population of Q is still in the reduced state when the back reaction (of Q^- with oxidized Z), assumed to lead to delayed light, has almost ended. Either Z^+ is indeed very stable, in which case one wonders why there was any delayed light initially, or an endogenous electron donor D_o^- on the oxygen evolving side of photosystem II is competing for the Z^+ quite successfully with Q^- in the reaction. In this respect D_o^- could be acting in the same manner as artificial electron donors such as hydroxylamine in quenching DLE (Bennoun, 46; Stacy et al., 47; Mohanty et al., 18).

Another complication is presented by the DCMU injection experiment (Figure 14). We see from Figure 10 that when unpoisoned chloroplasts are illuminated with weak light in the presence of HCO_3^-, very little variable fluorescence is observed. This may be explained by proposing a fast reoxidation of Q^- by A, a pool of electron acceptors between photosystems I and II, so that at no time does a large population of Q^- exist. Yet when the actinic light is terminated, and DCMU is injected into the chloroplast suspension, a large burst of DLE is seen (Figure 14) despite a seeming lack of one reactant, Q^-.

Clayton (39) explained the gush of DLE seen upon injection of DCMU as simply the result of a jump in the quantum yield of fluorescence, implying that there was no increase in the rate of charge recombination per se. However this idea does not explain why there is a lack of DLE gush when DCMU is added in the absence of HCO_3^-. To explain this phenomenon, we have postulated that in the absence of HCO_3^- the redox state of Z is inappropriate for the primary back reaction to occur. This will be true if oxidation states beyond S_1 (Z^+) are prohibited. DLE will be minimal regardless of the amount of Q^- present, or made available by DCMU injection. If in the presence of HCO_3^- oxidation states beyond S_1 are allowed (Z^{2+}, Z^{3+}), then DLE can occur given Q^-.

Still we are confronted with the problem of DLE occurring in the apparent absence of Q^-. We therefore repeat Lavorel's (40) suggestion that DCMU causes a shift in the proposed equilibrium; $Q^- + A \rightleftarrows Q + A^-$ to the left, thus making the electrons in the A pool also available for generating DLE.

The data obtained using unpoisoned chloroplasts indicates a more complex system. At high light intensity, the initial (0 → I) rise in the Chl a fluoresence transient (Figure 8) is more rapid in the absence of HCO_3^- while the final rise to steady state (p) is more rapid in the presence of HCO_3^-. The rise in fluorescence in the absence of HCO_3^- is associated with relatively little DLE (see Figure 13) while more DLE results in the presence of HCO_3^-.

A working model that we propose to explain the qualitative differences in the Chl a fluorescence transients in the presence and absence in of HCO_3^- is based on the following assumptions, some of which have already been mentioned:

(i) In the absence of HCO_3^-, reaction centers are initially (to a greater extent) in the S_o state (Z). (This is the only new assumption.) Upon illumination these centers can advance to the S_1 state (Z^+) but not to higher oxidation states.

(ii) There exists an endogenous electron donor D_o^- which can, in the absence of HCO_3^-, donate electrons to the S_1 state. Evidence for this donor is presented in chapter III.

(iii) In the presence of HCO_3^- some or most of the reaction centers are initially in the S_1 state (Z^+). Upon illumination, all centers can advance to all higher oxidation states ($S_o \rightarrow S_4$) consistent with the model of Kok (see Forbush et al., 41).

(iv) The overall fluorescence yield is a function of the redox state of the electron acceptor Q as first proposed by Duysens and Sweers (48; also see reference 49).

We shall consider first the chl a fluorescence transient observed in the absence of HCO_3^-. Assuming that a larger proportion of reaction centers are in the S_o state, illumination promptly causes these reaction centers to undergo the transition:

$$S_o \xrightarrow{h\nu} S_1; \ (\mathrm{ZChl\ Q} \xrightarrow{h\nu} \mathrm{Z^+Chl\ Q^-}) \qquad (3)$$

This accounts for the initial exponential rise in fluorescence (0 → I). The S_1 state is stable in the sense that it does not undergo a back reaction; little DLE is observed. However the reaction center will undergo reaction with A, the intersystem intermediate:

$$\mathrm{Z^+Chl\ Q^-} + \mathrm{A} \rightarrow \mathrm{Z^+Chl\ Q} + \mathrm{A^-} \qquad (4)$$

We assume the state Z^+Chl Q to be, in the absence of HCO_3^-, a nonphotochemically active quencher of fluorescence. Reaction (4) therefore should inhibit further rise in fluorescence and accounts for the I → D phase (see Munday and Govindjee, 50). We would expect that as reaction (4) proceeds, fluorescence should decrease to "0" level.

Instead fluorescence rises again (D → P). We, therefore, propose, again as a working hypothesis, that the following reaction takes place:

$$Z^{+}\text{Chl Q} + D_o^{-} \rightarrow \text{ZChl Q} + D_o \qquad (5)$$

This converts the reaction center to the initial state (S_o) allowing the cycle to begin again. Eventually, however, the A pool will become entirely reduced, causing the increased accumulation of fluorescent state $Z^{+}\text{Chl Q}^{-}$ and/or ZChl Q^{-}, hence the final D → P rise in fluorescence.

The Chl a fluorescence transient observed in the presence of HCO_3^- is that of the "normal" system. Assuming a large proportion of reaction centers are initially in the S_1 state, illumination causes the transition:

$$S_1 \rightarrow S_2 \; (Z^{+}\text{Chl Q} \overset{h\nu}{\rightarrow} Z^{2+}\text{ Chl Q}^{-}) \qquad (6)$$

The reaction centers undergo further reaction with A:

$$Z^{2+}\text{Chl Q}^{-} + A \rightarrow Z^{2+}\text{Chl Q} + A^{-} \qquad (7)$$

Reaction (6), however, is reversible to a greater extent than reaction (3) as indicated by the relatively larger amount of DLE observed in this case. Thus the rapid initial accumulation of a fluorescent state, $Z^{2+}\text{Chl Q}^{-}$ is inhibited by the action of Z^{2+} as well as A, both reoxidizing Q^-. The initial rise in fluorescence will therefore be slower compared to that seen in the absence of HCO_3^-. However, in the presence of HCO_3^-, all states formed after the reoxidation of Q^- are photochemically active. With continued illumination, all reaction centers proceed to higher oxidation states (back reactions notwithstanding). In doing so, the A pool is more rapidly filled than is possible when HCO_3^- is absent. The subsequent accumulation of Q^- accounts for the later more rapid rise of fluorescence to the steady state (P).

Explaining all fluorescence transient data obtained thus far in various laboratories on the basis of the oxidation-reduction states of the photosystem II reaction centers is an oversimplification. One may still need to consider complications due to energy migration among pigment system II units (see Lavorel and Joliot, 51), effects on membrance conformation (see Lavorel, 40), spillover among photosystems I and II (see Govindjee and Papageorgiou, 49) and other factors. Delayed light emission is also a very complex phenomenon and explaining it solely on the basis of oxidation-reduction states of the reaction centers likewise may be an oversimplification. Nevertheless, such an interpretation seems to us to be the simplest way of understanding, at present, the complex data presented here.

A test of the particular hypothesis presented here would be to measure the ratio of S_o and S_1. Preliminary experiments by B. Kok (unpublished observations) and by Stemler _et al_. (36) indicate that the absence of HCO_3^- may have slightly increased the S_o/S_1 ratio. However, further experiments are needed to accept or reject the various aspects of the above hypothesis.

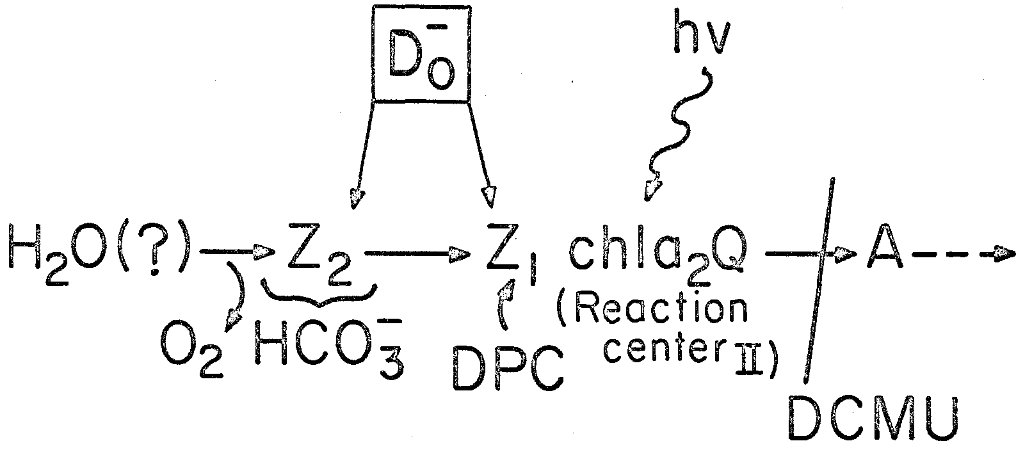
VI: CONCLUDING REMARKS: POSSIBLE ROLES FOR BICARBONATE IN THE OXYGEN EVOLVING MECHANISM

The results presented in the preceding chapters allow the conclusion that HCO_3^- is necessary for oxygen evolution and that its site of action is near the oxygen evolving steps (Figure 16). Left unanswered is the question of the role of this ion in oxygen evolution. Why is there the need for this specific ion?

A. Source of O_2 in Photosynthesis?

According to Warburg's theory (1, see also 52), CO_2 acts as substrate in the oxygen evolving reaction. That is, CO_2 is the source of the evolved oxygen. This idea, as mentioned in the introduction has been almost universally rejected. The evidence upon which Warburg's theory is discounted was provided by the experiments of Ruben *et al.* (53). Using ^{18}O, these workers showed that the isotopic composition of oxygen evolved by Chlorella cells was comparable to that of the suspension water, whether the isotope was supplied in the form of $H_2{}^{18}O$ or $C^{18}O_2$. These results, however, can be explained on the basis of isotopic exchange. ^{18}O is readily transferred between H_2O and CO_2, which chemically interact. To retard this exchange of ^{18}O, the external pH was kept high. In fact, Ruben *et al.* showed that very little isotopic exchange was possible under such conditions in the external medium. However, since whole Chlorella cells were used, one may question the assumption that their internal pH reflected the external pH, and therefore no exchange took place within the cells. Gas collection times were long, over an hour in most experiments. Warburg (52) also

Figure 16. A scheme for Photosystem II. Light ($h\nu$) is absorbed by the photosystem II reaction centers (Z, chl a_2 O_2). Z, is the electrically neutral primary reluctant, Q is the primary oxidant. After the initial charge separation occurs and O_2 is reduced, the electron passes to a pool of electron acceptors (A) between photosystems I and II unless blocked by DCMU. Diphenyl carbazide (DPC) or an endogenous electron donor (D_o^-) can provide electrons to the then oxidized Z, keeping it electrically neutral. Bicarbonate (HCO_3^-) permits the formation of, or stabilizes reactions centers which netain their positive change. Thus the reaction centers can accumulate the 4 positive charges needed to evolve oxygen.


D_0^-
hv
H_2O(?)
Z_2
Z_1
chla_2Q
(Reaction center II)
A
O_2
HCO_3^-
DPC
DCMU

pointed out that Chlorella contain carbonic anhydrase, as do many, if not all plants (54). This enzyme would certainly speed exchange within cells. Despite the ambiguities presented by these experimental conditions the work of Ruben et al. has been accepted as proof that oxygen evolves from H_2O, and not CO_2 as Warburg insisted. Since the completion of these experiments, done in 1941, no additional direct evidence of any kind has been discovered to support the theory that oxygen evolves from H_2O. It can be viewed with some wonder then, how the idea that oxygen originates in H_2O has gradually gone from hypothesis to theory to undisputed fact. It is the opinion of this author that the evidence supporting the theory that oxygen originates in H_2O is simply too meager and too weak to warrant such unqualified acceptance at this time. For a review of experiments related to this controversy see Rabinowitch (55).

On the other hand, there is no direct evidence that oxygen comes from CO_2 either. True, HCO_3^- is needed for oxygen evolution, but it need not be as substrate in the reaction. To settle unequivocally the controversy concerning the origin of molecular oxygen, we plan, in the near future, to repeat the ^{18}O experiments using HCO_3^- depleted chloroplasts rather than whole cells. The isotope would be supplied as $HC^{18}O_3^-$ under conditions such that oxygen evolution is known to be dependent on exogenous HCO_3^-. If the percent of isotope in the oxygen evolved does not reflect that of the supplied $HC^{18}O_3^-$ when this ion is, in fact, stimulating oxygen evolution, we can be very sure that HCO_3^- is only acting "catalytically" and that Warburg is wrong in saying that HCO_3^- is a substrate in oxygen evolution. Until this experiment is done, however, we may be wise to keep an open mind in regard to Warburg's view of the role of HCO_3^- in oxygen evolution.

B. HCO_3^- As a Regulatory Ion

There is a possibility that HCO_3^- is acting as part of a control mechanism, i.e., an allosteric effector of the oxygen evolving enzyme. It is very reasonable to argue that if little or no CO_2 is present to be fixed by the "dark reactions" of photosynthesis, there is no need for the reducing power and ATP formed in the "light reactions" and hence no need to evolve oxygen. However, an allosteric control mechanism requires a loosely bound effector. If the effector is not easily removable, the controlled enzyme cannot respond rapidly to varying concentration of the effector. Assuming that HCO_3^- were acting as an allosteric effector, it is of some concern that rather rigorous treatment is necessary in order to remove it from broken chloroplasts. Still, this difficulty alone cannot exclude a controlling mechanism as a possible role of HCO_3^- in oxygen evolution.

C. HCO_3^- and Membrane Phenomena

The fact that HCO_3^- affects almost every phenomenon associated with photosystem II activity suggests that it may have some effect on the membrance (e.g., membrane potential). A thorough study of this possibility has not yet been made. Some preliminary experiments (A. Stemler, unpublished results) using nigericin, gramicidin D and valinomycin show that these substances do not modify in any way the bicarbonate effect observed. However, it cannot be overemphasized that these studies were cursory at best and firm conclusions cannot be made at this time. Membrane potential may still be the means by which HCO_3^- exerts its influence.

HCO_3^- may be causing small conformational changes in membrane protein and thus influencing oxygen evolution. This would be expected, in fact, if it were acting as an allosteric effector. The glutaraldehyde experiment reported here (Chapter IV) rules out the possibility that HCO_3^- is responsible for more massive configurational changes in the membrane to stimulate oxygen evolution. So far it has not been possible to determine if HCO_3^- is causing small conformational changes. (Experiments are in progress, in our laboratory, to answer these questions.)

D. HCO_3^- and the "S" States

Finally, we have examined the possibility that HCO_3^- is influencing oxygen evolution by facilitating the formation or stability of higher oxidation states of the reaction centers according to the kinetic model of Forbush _et_. _al_. (41). Preliminary studies (36) measuring oxygen evolution in response to brief light flashes, show that HCO_3^- is, in fact, accelerating the formation of higher oxidation states of the reaction centers. Interestingly, the dark deactivation of these states appear unaffected by HCO_3^-. (A more detailed report is not yet possible as these studies are still in progress.)

The fact that HCO_3^- affects the "S" states of photosystem II, does not, however, really answer why HCO_3^- is necessary. None of the possible roles of HCO_3^- as discussed here has been completely eliminated, nor are they mutually exclusive. For this reason this work cannot be viewed as definitive, rather it should be seen as a starting point for a more intense investigation of the role of HCO_3^- in photosynthetic oxygen evolution.

[A large portion of the data presented in this thesis has been published already (56, 57) or is in press (58). (A summary of my work appears in reference 59.) It was considered necessary to assure priority of my work by submitting it for publication prior to the writing of this thesis. However, this thesis has not been published *in toto*.]

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(2) Bicarbonate Ion as a Critical Factor in Photosynthetic Oxygen Evolution, Plant Physiology, 52, 119-123 (1973).

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