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The Effect of Bicarbonate on Photosynthetic Oxygen Evolution in Flashing Light in Chloroplast Fragments

(photosynthesis/Hill reaction/photochemical reactions of system II)

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ABSTRACT The ability of bicarbonate ion (HCO_3^-) to stimulate photosynthetic oxygen evolution in maize chloroplast fragments exposed to continuous light depends on light intensity. Stimulation by HCO_3^- is less at low intensities. In HCO_3^--depleted chloroplasts exposed to brief saturating light flashes, period 4 oscillations (in O_2 yield per flash) are damped within three cycles. Readdition of HCO_3^- to these preparations restores the oscillatory pattern to higher flash numbers, indicating that HCO_3^- reduces the probability of "misses" in the photosystem II reaction center. The rate of the dark relaxation reaction $S_n' \dashrightarrow S_{n+1}$ (where S refers to the oxidation state of the oxygen-evolving mechanism and n = 0, 1, or 2), after a photoact in the photosystem II reaction center, is retarded in HCO_3^--depleted chloroplasts compared to the rate for this reaction in depleted chloroplasts to which HCO_3^- has been resupplied. However, the final oxygen-evolving reaction after the accumulation of four positive charges appears to be independent of HCO_3^-. Bicarbonate has no effect on the dark deactivation of the higher oxidation states (S_2 and S_3) of the positive charge-accumulating system. We propose two alternate ways in which the kinetic model of oxygen evolution developed by Kok *et al.* [(1970) *Photochem. Photobiol.* 11, 457–475] can be extended to include the action of HCO_3^-.

Recent investigation of the role of HCO_3^- in the Hill reaction indicates that this ion plays a critical role in the oxygen-evolving mechanism (1–3). Evidence is available that strongly suggests that HCO_3^- acts on the oxygen-evolving side of photosystem (PS) II. Electron flow from the artificial electron donor diphenyl carbazide to dichlorophenolindophenol via PS II is insensitive to HCO_3^- (1). Effects of HCO_3^- on chlorophyll (Chl) *a* fluorescence transients and on delayed light emission in the 0.5- to 5-sec time period also seem to suggest a site of action of HCO_3^- on the oxygen-evolving side of PS II (2). This latter work led Stemler and Govindjee (2) to speculate that HCO_3^- somehow stabilized higher oxidation states of the PS II reaction centers (referring to the kinetic model of oxygen evolution of Kok *et al.*, ref. 4). We therefore studied the effects of HCO_3^- on oxygen evolution in response both to continuous light and to brief light flashes. Our results show that HCO_3^- reduces the frequency of reaction center "misses" and thus maintains oscillations in oxygen yield per flash for a much greater number of flashes. In addition, HCO_3^- is shown here to accelerate the relaxation reactions ($S_n' \dashrightarrow S_{n+1}$, where n = 0, 1, or 2) after a photoact. The final oxygen-evolving reaction ($S_4 + O_2$ precursor(s) $\dashrightarrow O_2 + S_0$), however, appears to be independent of HCO_3^-.

Abbreviations: Chl, chlorophyll; PS II, photosystem II; Z Chl a_2 Q, reaction center of PS II, Z and Q being electron donor and acceptor, respectively, and Chl a_2 being the reaction center chlorophyll (the *primary* electron donor) of PS II.

METHODS

Chloroplast Preparation. Maize (*Zea mays*) chloroplasts were obtained in a manner already described (1). While even under optimum conditions maize chloroplasts usually do not perform the Hill reaction at very high rates compared to chloroplasts from other sources, we continue to use maize to minimize precipitation of the chloroplasts during the HCO_3^--depletion procedure (3). However, HCO_3^--depletion of pea (*Pisum sativa*) chloroplasts (T. Wydrzynski; unpublished data) under milder conditions, produced 4- to 10-fold HCO_3^- stimulation of oxygen evolution with total yield equal to untreated controls. The HCO_3^--depletion procedure, therefore, does not necessarily result in gross chloroplast damage, thereby accounting in some way for the HCO_3^- effect. To deplete the chloroplasts of HCO_3^- we suspended them in a solution containing 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer at pH 5.0. The suspension was stirred slowly for 30 min at room temperature while the gas above the suspension was continuously flushed with nitrogen. This treatment proved somewhat less damaging to activity than bubbling N_2 directly through the suspension as in previous work (1). After the chloroplasts were depleted of HCO_3^-, they were centrifuged in capped test tubes that had been flushed with N_2, and resuspended in reaction mixture. Reaction mixtures are described in the figure legends. All vessels and reaction mixtures were carefully sealed or otherwise handled to avoid contamination with atmospheric CO_2 prior to deliberate addition of $NaHCO_3$.

Steady-State O_2 Evolution. To measure oxygen evolution in continuous light, we used a Clark-type electrode (Yellow Springs Oxygen Monitor, model 53). The signal was recorded by an Esterline Angus (model E11015) recorder. Rates of oxygen evolution were calculated from the slope of the recorder trace during the first minute of illumination. Samples were illuminated with a GE 120 V, 650 W, DVY lamp. The beam passed through 15 cm of water and a Corning C.S. 3–71 yellow cut-off filter before striking the sample. The sample holder was a cylinder having a diameter of about 1 cm and total capacity of 1.7 ml. Incident intensity was 5×10^5 ergs·cm^{-2}·sec^{-1} or reduced from this value by means of calibrated neutral density filters. Samples were initially anaerobic.

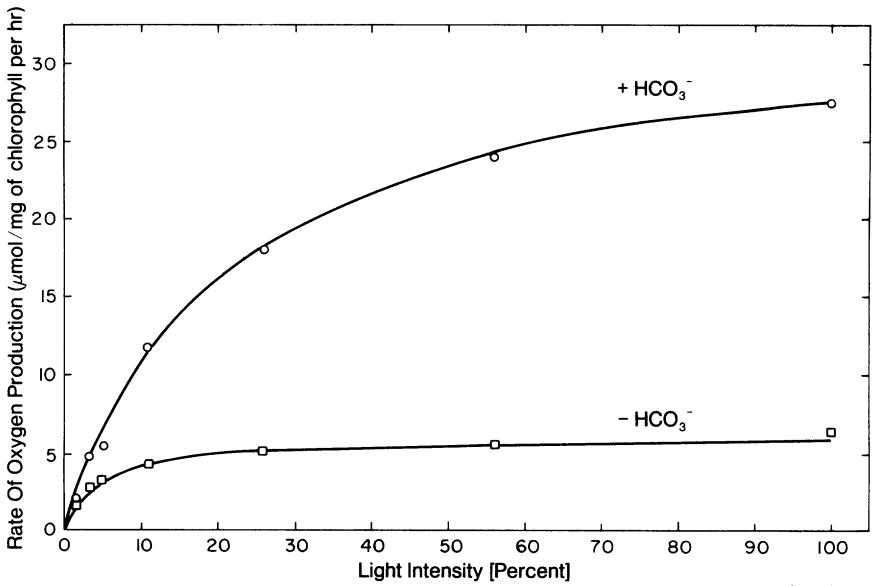

FIG. 1. Rate of oxygen evolution in continuous light as a function of light intensity. The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer at pH 6.8; 1 mM potassium ferricyanide; ±0.01 M $NaHCO_3$; and 50 μg of Chl·ml^{-1}. The light intensity was 5 × 10^5 ergs·cm^2·sec^{-1} or reduced by neutral density filters. A Corning C.S. 3-71 (yellow) cut-off filter was used. Initially the conditions were anaerobic. Each point represents an average of five measurements.

O_2 Evolution in Flashing Light. The apparatus used for measuring oxygen evolution in response to brief light flashes was described by Weiss and Sauer (5) and modified according to Babcock (6). The xenon lamp pulses were 10 μsec in duration and were filtered through Corning 1-69 and 3-74 filters before being focused on the electrode surface. All flashes used in these experiments were of saturating intensity. The solution flowing above the membrane holding the chloroplasts to the surface of the platinum electrode contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer at pH 6.8 and was either HCO_3^--free or supplied with 0.01 M $NaHCO_3$ in bicarbonate re-addition experiments. The electrolyte was gassed continuously with 80% N_2–20% O_2.

RESULTS

Oxygen evolution in continuous light: An intensity curve

Oxygen evolution, with chloroplasts previously depleted of HCO_3^-, was measured as a function of light intensity (Fig. 1). It is clear that the stimulation caused by resupplying 0.01 M $NaHCO_3$ to the chloroplasts depends on light intensity. At saturating intensity stimulation is nearly 5-fold. This stimulation declines with intensity so that, at the lowest intensity used, it is less than 2-fold.

Oxygen evolution in response to brief saturating light flashes

Effects of HCO_3^- on Oscillations in Oxygen Yield. The kinetics of oxygen evolution in response to brief light flashes have already been described in detail for normal systems by Joliot *et al.* (7) and Kok *et al.* (4) (also see Mar and Govindjee, ref. 8). From the evidence presented in Fig. 2 (bottom), HCO_3^--depleted chloroplasts, when normalized to the same total oxygen yield (under steady state), show damped oscillations in oxygen evolution as a function of flash number compared to those chloroplasts resupplied with 0.01 M HCO_3^-. (In HCO_3^--depleted chloroplasts resupplied with HCO_3^-, oscillations were very similar to untreated controls; data not shown.) The damping of oscillations in the HCO_3^--depleted chloroplasts is suggested to be due to a greater number of "misses." Reducing the number of misses is not the only function of HCO_3^-, however, as will be seen from other results to be discussed below.

It is also evident from the recorder traces presented in Fig. 2 (top) that total oxygen yield induced by light flashes spaced 1 sec apart is nearly 2-fold greater in the presence of HCO_3^-. Under high-intensity continuous light, these same chloroplasts showed a 4- to 5-fold greater rate of oxygen evolution. Thus, brief flashes of high-intensity light spaced 1 sec apart produce the same reduced HCO_3^- stimulation as seen with low-intensity continuous light (Fig. 1). However, a 50% decrease in steady oxygen yield in the absence of HCO_3^- cannot be attributed to misses alone. If the miss rate were indeed 50%, the yield on the third flash would be much less than that of the steady-state yield. Since it is not, a miss rate of less than 20% is implied. We must, therefore, propose that a certain percentage of reaction centers are completely inactive in the absence of HCO_3^- and that this, even more than an increase in the miss rate, lowers the steady-state yield about 50%.

Effect of HCO_3^- on Relaxation Reactions ($S_n' \rightarrow S_{n+1}$). Studies on the rates of the dark relaxation reactions occurring between photoacts have been made by Kok *et al.* (4), and particularly by Bouges-Bocquet (9). The rates are measured by varying the time between the flashes and measuring the effect on the final yield of O_2. The half-times of the reactions $S_0' \xrightarrow{\text{dark}} S_1$, $S_1' \xrightarrow{\text{dark}} S_2$, and $S_2' \xrightarrow{\text{dark}} S_3$ are all in the order of 200–600 μsec in normal chloroplasts (9). In HCO_3^--depleted chloroplasts, however, the half-times of these reactions are dramatically extended, while resupplying HCO_3^- restores the normal rates. For example, Fig. 3 shows the effect of HCO_3^- on the reactions, $S_2' \rightarrow S_3$ [this process proceeds biphasically and may involve two or more reactions (9)]. The half-time for this reaction is about 11 msec in HCO_3^--depleted chloroplasts and about 700 μsec in HCO_3^--depleted chloro-

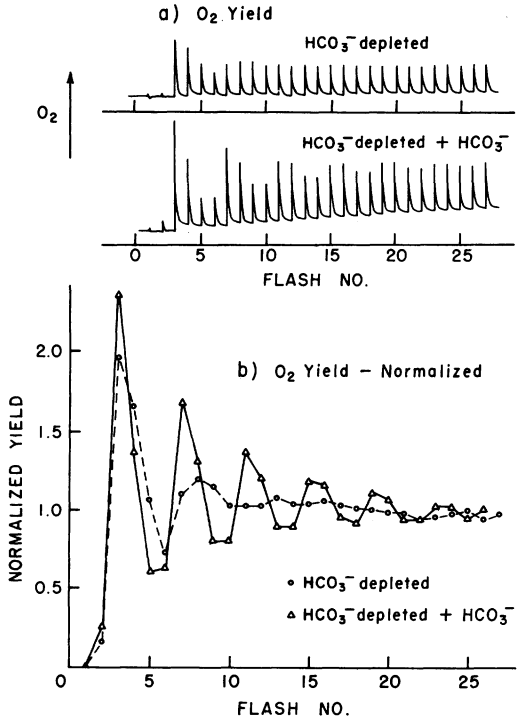

FIG. 2. Oxygen evolution in flashing light in the presence and absence of 0.01 M $NaHCO_3$ after 5 min in the dark. (Top) Recorder traces; saturating 10-μsec flashes spaced 1 sec apart were used to stimulate oxygen evolution. (Bottom) Oxygen yield as a function of flash number, from experimental traces in (a), normalized to the same total steady-state yield of oxygen. The chloroplast suspension injected onto the platinum electrode contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer (pH 6.8), 20 $\mu g \cdot ml^{-1}$ of ferredoxin, 0.5 mM $NADP^+$, and 0.3 mg of $Chl \cdot ml^{-1}$. The chloroplasts used had been depleted of HCO_3^-. To resupply HCO_3^- to the chloroplasts, HCO_3^- (to 10 mM) was added to the electrolyte flowing over the membrane holding the chloroplasts to the platinum. Other conditions are described in *Methods*.

plasts resupplied with HCO_3^-. Thus, HCO_3^- speeds the rate of this reaction, $S_2' \rightarrow S_3$, by more than 10-fold.

The rate of the reaction, $S_1' \rightarrow S_2$, is affected in a manner similar to that for $S_2' \rightarrow S_3$. This is shown in Fig. 4. Again, the half-time is about 10 msec in HCO_3^--depleted chloroplasts and about 600 μsec in chloroplasts resupplied with HCO_3^-. Likewise, the rate of the reaction $S_0' \rightarrow S_1$, calculated by the method of Bouges-Boquet (9), is comparably reduced in HCO_3^--depleted chloroplasts (data not shown).

The final [and slowest (9)] relaxation reaction(s), $S_3' \rightarrow S_4 + O_2$ precursor(s) $\rightarrow S_0 + O_2$, occurs with the release of oxygen. The rate of O_2 production was monitored directly (Fig. 5). Unfortunately, the method is limited by the response time of the instrument. The main factor is the diffusion-limited time between oxygen evolution and contact of the dissolved gas with the platinum electrode surface. This time is about 6 msec for our electrode system. As shown in Fig. 5, oxygen evolution, after a flash, from untreated, HCO_3^--depleted and HCO_3^--depleted chloroplasts resupplied with HCO_3^- is detected with the instrument-limited time of 6 msec. Therefore, the reactions, $S_3' \rightarrow S_4$ and $S_4 + O_2$ precursor(s) $\rightarrow S_0 + O_2$, proceed with half-times less than or equal to 6 msec in HCO_3^--depleted chloroplasts. This instrument-limited time is in marked contrast to the measured half-

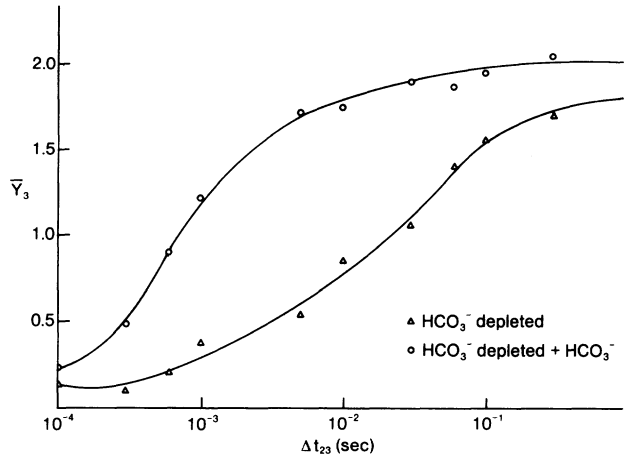

FIG. 3. Time course of the relaxation reaction $S_2' \rightarrow S_3$; oxygen yield on the third flash, $\bar{Y}_3$, in the presence and absence of 0.01 M $NaHCO_3$ as a function of the time between the second and third flash (Δt_{23}). $\bar{Y}_3$ is the yield normalized with respect to the steady-state yield. Other flashes are 1 sec apart. Other conditions are as in the legend of Fig. 2.

times of 10–12 msec for the reactions, $S_n' \rightarrow S_{n+1}$ ($n = 0$, 1, or 2), in these same HCO_3^--depleted chloroplasts (Figs. 3 and 4) and indicates that the final oxygen-yielding reactions are very probably independent of HCO_3^-.

Effect of HCO_3^- on the Deactivation of the States S_2 and S_3. If the time between the first and second light flash, or between the second and third, is extended beyond about 1 sec, deactivation of the states S_2 or S_3, respectively, can be observed (10, 11). Fig. 6 shows that the decay of the S_3 state in chloroplasts depleted of HCO_3^- and those resupplied with HCO_3^- follow the same time course, although there is a difference in the amount of O_2 evolved. Likewise, the decay of the S_2 state (data not shown) is the same in the presence and absence of HCO_3^-. It is clear, therefore, that HCO_3^- has no effect on the stability of the higher oxidation states (i.e., S_2 and S_3) of the PS II positive-charge accumulating system, but only on the rate of formation of these states after a photoact (Figs. 3 and 4).

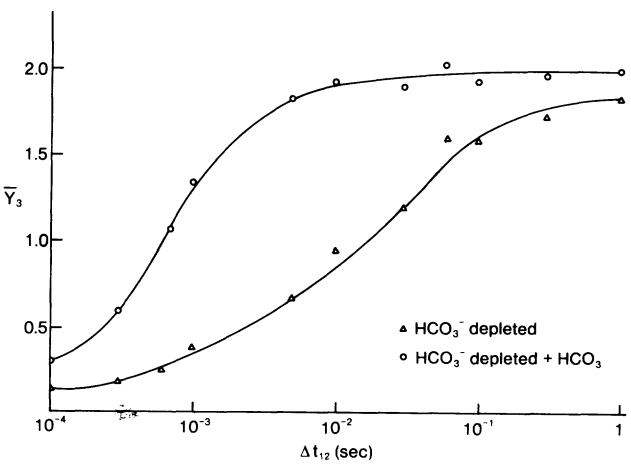

FIG. 4. Time course of the relaxation reaction, $S_1' \rightarrow S_2$; oxygen yield on the third flash, $\bar{Y}_3$, in the presence and absence of 0.01 M $NaHCO_3$ as a function of the time between the first and second flash (Δt_{12}). Other conditions are as in the legend of Fig. 3.

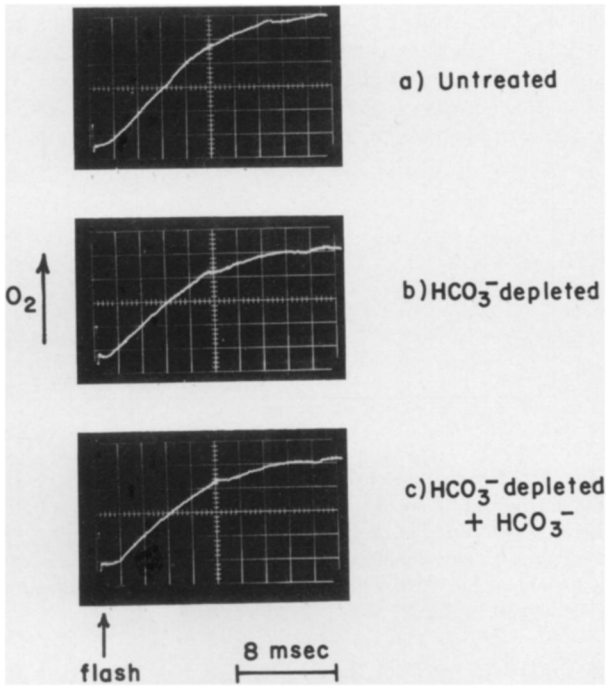

FIG. 5. Oxygen evolution after a light flash with (a) untreated, (b) HCO_3^--depleted, and (c) HCO_3^--depleted chloroplasts resupplied with 0.01 M $NaHCO_3$. Other conditions are as in the legend of Fig. 3.

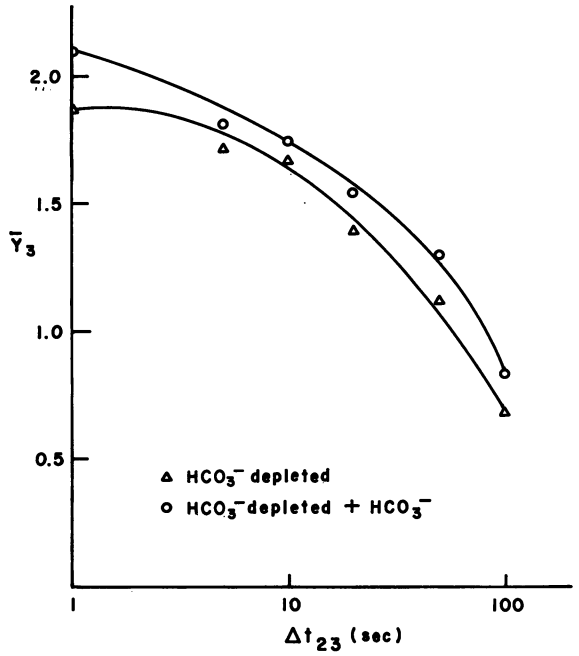

FIG. 6. Decay of the S_3 state; oxygen yield on the third flash, $\bar{Y}_3$, in the presence and absence of 0.01 M $NaHCO_3$ as a function of the time between the second and third flash (Δt_{23}). Other conditions are as in the legend of Fig. 3.

DISCUSSION

The intensity curves presented in Fig. 1 confirm the findings of Good (12) and also Izawa (13). Both observed less stimulation of oxygen evolution (measured manometrically) by HCO_3^- at low light intensity. At the same time, these results seemingly contradict earlier findings of Stemler and Govindjee (3), who measured ferricyanide reduction amperometrically, and West and Hill (14) who measured dichlorphenoloindophenol reduction spectrophotometrically. These workers showed the HCO_3^- effect to be independent of light intensity. It is difficult to reconcile these apparently contradictory results, since they imply different mechanisms of action of HCO_3^-. A light-intensity-dependent effect implies that HCO_3^- is acting on "dark," probably enzymatic, reactions, while a light-intensity-independent effect implies that HCO_3^- is affecting "photochemical" processes.

Our present knowledge of the mechanism of oxygen evolution may be useful in explaining these apparent contradictions. We now know that oxygen is evolved by a reaction center after a series of four photoacts and at least that number of alternating dark reactions (15). A factor that affects the rate of a dark reaction may, in fact, also affect the yield of a subsequent photoact and vice versa. It would seem difficult to tell, therefore, from intensity curves alone whether a "light" or "dark" reaction is being affected, especially if we measure only the final product, i.e., oxygen or reduced Hill oxidant. It appears from Fig. 1 that HCO_3^- is speeding "dark" reactions, and the other data presented here provide more convincing evidence that this is the case. Yet, in doing so, HCO_3^- is also facilitating "light" reactions.

The observation that HCO_3^- speeds the relaxation reactions between photoacts (Figs. 3 and 4) explains why less HCO_3^- stimulation is seen at low light intensity (Fig. 1) and when saturating flashes are given spaced 1 sec apart. Under these conditions the reaction centers have enough time to undergo relaxation (even at the lower rate imposed by HCO_3^- depletion) before another photon arrives. Thus, HCO_3^- has less observed effect. The small stimulation in O_2 yield per flash (Fig. 2, top) that is still observed under these conditions must be due primarily to a greater number of active reaction centers and secondarily to the reduced number of "misses" that occur in the presence of HCO_3^-.

The ability of HCO_3^- to speed relaxation reactions can be interpreted to indicate either that this ion accelerates the reoxidation of the primary electron acceptor for PS II by the pool of intersystem intermediates, or that HCO_3^- is acting on the oxygen-evolving side of PS II. The first interpretation, as mentioned in the introduction, is inconsistent with our previous work (1–3). For example, if HCO_3^- depletion imposed a block between Q and A, one would predict that the chlorophyll *a* fluorescence transient would rise to maximum (F_∞) very fast, as it does in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Actually, the rise to the steady-state fluorescence level is much slower in the HCO_3^--depleted chloroplasts (2). Further evidence that HCO_3^- is not acting on the reducing side of PS II is provided by long-term delayed light emission, which is thought to reflect back reactions after light-induced charge separation. If HCO_3^- accelerated the reoxidation of Q by A, less Q^- should be available to back react. Hence, we would expect less delayed light emission in the presence of HCO_3^-. Instead, more delayed light emission is observed in the presence of HCO_3^- (2). These results, the absence of a HCO_3^- effect when PS II electron flow is from diphenylcarbazide → dichloroindophenol, and others already discussed (1–3), strongly imply that HCO_3^- is acting on the oxygen-evolving side of PS II. However, since all our arguments that support this view are admittedly based on various assumptions, we plan to continue to test this hypothesis.

Meanwhile, it appears reasonable to consider how HCO_3^- is influencing the oxygen-evolving mechanism.

Working Hypotheses. The kinetic model for oxygen evolution advanced by Kok *et al.* (4) can now be extended in several possible ways to include the action of HCO_3^-. Ignoring for our purposes the reducing (Q) side of PS II, we may represent a photoact as: Chl $a_2 \xrightarrow{h\nu}$ Chl a_2^+, where Chl a_2 is the reaction center pigment, and the primary electron donor to Q (16). This undergoes reaction with the electron donor Z: Chl a_2^+ + Z $\rightarrow$ Chl a_2 + Z^+. Z^+, in turn, undergoes the HCO_3^--mediated reaction: $Z^+ + S_n \xrightarrow{HCO_3^-} Z + S_{n+1}$, where S is the charge accumulating enzyme or system in the nth state (n = 0, 1, or 2) and $Z^+ + S_n$ corresponds to the S_n' state mentioned earlier in *Results*. Since the final oxygen-evolving reaction(s) appears to be independent of HCO_3^-, we can imagine it occurs simply as: $Z^+ + S_3 \rightarrow S_4$; $S_4 + O_2$ precursor(s) $\rightarrow S_0 + O_2$. A second possibility is that Z^+ and S_3 cooperate as: $Z^+ + S_3 + O_2$ precursor(s) $\rightarrow Z + S_0 + O_2$.

Thus, in the above model, HCO_3^- controls the transfer of the first three electrons from the positive charge-accumulating mechanism to oxidized Z.

An alternative explanation of the HCO_3^- effect is also possible. In this second model Z is eliminated as an intermediate entirely, or rather it is equated with the positive charge-accumulating system. Thus, the reaction sequence can be written as: Chl $a_2 \xrightarrow{h\nu}$ Chl a_2^+; Chl $a_2^+ + S_n \xrightarrow{HCO_3^-}$ Chl $a_2 + S_{n+1}$, where n is again equal to 0, 1, or 2. In this model HCO_3^- controls the rate of transfer of the first three electrons from the positive charge-accumulating system (called S in the model of Kok *et al.*) directly to the oxidized reaction center Chl a_2^+.

Besides accelerating the relaxation reactions, HCO_3^- also reduces the number of misses that are apt to occur in reaction centers (Fig. 2). While these are clearly different effects, they are not necessarily independent. We propose that if the relaxation reactions after a photoact are accelerated by HCO_3^-, less time might be available for a back reaction of Chl a_2^+ and Q^-. Such a back reaction, occurring in the msec time period or earlier after a flash, could constitute a miss. It follows that this reaction would have less time to occur in the presence of HCO_3^-. If this is the case, we might expect greater amounts of delayed light emission (reflecting more misses) from HCO_3^--depleted systems in the msec time range after a flash.

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1. Stemler, A. & Govindjee (1973) *Plant Physiol.* **52,** 119–123.
2. Stemler, A. & Govindjee (1973) *Photochem. Photobiol.* **19,** 227–232.
3. Stemler, A. & Govindjee (1974) *Plant Cell Physiol.* **15,** 533–544.
4. Kok, B., Forbush, B. & McGloin, M. (1970) *Photochem. Photobiol.* **11,** 457–475.
5. Weiss, C. & Sauer, K. (1970) *Photochem. Photobiol.* **11,** 495–501.
6. Babcock, G. T. (1973) *Kinetics and Intermediates in Photosynthetic Oxygen Evolution,* Ph.D. Dissertation, University of California, Berkeley. LBL-2172.
7. Joliot, P., Barbieri, G. & Chabaud, R. (1969) *Photochem. Photobiol.* **10,** 309–329.
8. Mar, T. & Govindjee (1972) *J. Theor. Biol.* **36,** 427–446.
9. Bouges-Bocquet, B. (1973) *Biochim. Biophys. Acta* **292,** 772–785.
10. Joliot, P., Joliot, A., Bouges, B. & Barbieri, G. (1970) *Photochem. Photobiol.* **14,** 287–307.
11. Forbush, B., Kok, B., & McGloin, M. (1971) *Photochem. Photobiol.* **14,** 307–321.
12. Good, N. E. (1963) *Plant Physiol.* **38,** 298–304.
13. Izawa, S. (1962) *Plant Cell Physiol.* **3,** 221–227.
14. West, J. & Hill, R. (1967) *Plant Physiol.* **42,** 819–826.
15. Joliot, P. & Kok, B. (1975) in *Bicenergetics of Photosynthesis,* ed. Govindjee (Academic Press, New York), in press.
16. Butler, W. L. (1973) *Accounts Chem. Res.* **6,** 177–184.