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. Readmission 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 $S'_n\rightarrow S_{n+1}$ (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 ($S_0$ and $S_1$) 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 ($S'_n\rightarrow S_{n+1}$, where $n = 0, 1, or 2$) after a photoact. The final oxygen-evolving reaction ($S_0 + O_2$ precursor(s) $\rightarrow O_2 + S_0$), however, appears to be independent of HCO$_3^-$.

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 sativum) 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 E1015) 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, 12V 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 $5 \times 10^6$ ergs cm$^{-2}$ sec$^{-1}$ or reduced from this value by means of calibrated neutral density filters. Samples were initially anaerobic.

Abbreviations: Chl, chlorophyll; PS II, photosystem II; Z Chl $\alpha_1$, Q, reaction center of PS II; Z and Q being electron donor and acceptor, respectively, and Chl $\alpha_1$ being the reaction center chlorophyll (the primary electron donor) of PS II.
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\[ \text{HCO}_3^- \]

8). ref. HC03--depleted chloroplasts, light total in the presence of HC03--free with NaHCO3 to the chloroplasts depends on light 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 HCO3--free or supplied with 0.01 M NaHCO3 in bicarbonate re-addition experiments. The electrolyte was gassed continuously with 80% N2-20% O2.

\[ \text{O}_2 \text{ Evolution in Flashing Light.} \] 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 HCO3--free or supplied with 0.01 M NaHCO3 in bicarbonate re-addition experiments. The electrolyte was gassed continuously with 80% N2-20% O2.

RESULTS

Oxygen evolution in continuous light: An intensity curve

Oxygen evolution, with chloroplasts previously depleted of HCO3--, was measured as a function of light intensity (Fig. 1). It is clear that the stimulation caused by resupplying 0.01 M NaHCO3 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 HCO3-- 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), HCO3--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 HCO3--. (In HCO3--depleted chloroplasts resupplied with HCO3--, oscillations were very similar to untreated controls; data not shown.) The damping of oscillations in the HCO3--depleted chloroplasts is suggested to be due to a greater number of "misses." Reducing the number of misses is not the only function of HCO3--, 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 HCO3--. 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 HCO3-- stimulation as seen with low-intensity continuous light (Fig. 1). However, a 50% decrease in steady oxygen yield in the absence of HCO3-- 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 HCO3-- and that this, even more than an increase in the miss rate, lowers the steady-state yield about 50%.

Effect of HCO3-- on Relaxation Reactions (S0' \( \rightarrow \) S+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-Boquet (9). The rates are measured by varying the time between the flashes and measuring the effect on the final yield of O2. The half-times of the reactions S0' \( \rightarrow \) S+1, S+1 \( \rightarrow \) S2, and S2 \( \rightarrow \) S3 are all in the order of 200-600 μsec in normal chloroplasts (9). In HCO3--depleted chloroplasts, however, the half-times of these reactions are dramatically extended, while resupplying HCO3-- restores the normal rates. For example, Fig. 3 shows the effect of HCO3-- on the reactions, S0' \( \rightarrow \) S1 [this process proceeds biphasically and may involve two or more reactions (9)]. The half-time for this reaction is about 11 msec in HCO3--depleted chloro-
plasts resupplied with HCO$_3^-$.

Thus, HCO$_3^-$ speeds the rate of this reaction, S$_7' \rightarrow$ S$_9$, by more than 10-fold.

The rate of the reaction, S$_7' \rightarrow$ S$_9$, is affected in a manner similar to that for S$_3' \rightarrow$ S$_5$. This is shown in Fig. 4. Again, the half-time is about 10 msec in HCO$_3^-$-depleted chloroplasts and about 600 $\mu$s in chloroplasts resupplied with HCO$_3^-$. Likewise, the rate of the reaction S$_7' \rightarrow$ S$_9$, 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$_7' \rightarrow$ S$_9$ + O$_2$ precursor(s) $\rightarrow$ S$_9$ + 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$_7' \rightarrow$ S$_9$ and S$_9$ + O$_2$ precursor(s) $\rightarrow$ S$_9$ + 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. 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; a saturated 10-msec 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 mL$^{-1}$ of ferredoxin, 0.5 mM NADP$^+$, and 0.3 mg of Chl 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.

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$_3$ and S$_4$. 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$_3$ or S$_4$, 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$_4$ 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$_3$ and S$_4$) 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. 3. Time course of the relaxation reaction S$_4' \rightarrow$ S$_4$; oxygen yield on the third flash, Y$_3$, in the presence and absence of 0.01 M NaHCO$_3$ as a function of the time between the third and second flashes (A$_{32}$). Y$_3$ is the yield normalized with respect to the steadystate yield. Other flashes are 1 sec apart. Other conditions are as in the legend of Fig. 2.

Fig. 4. Time course of the relaxation reaction S$_5' \rightarrow$ S$_5$; oxygen yield on the third flash, Y$_5$, in the presence and absence of 0.01 M NaHCO$_3$ as a function of the time between the first and second flashes (A$_{34}$). 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.

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 dichlorophenolindophenol 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 "missed" events 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 (Fm) 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 dihydropyranobacizide to dichlorophenolindophenol, 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 $\text{HCO}_3^-$ 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 $\text{HCO}_3^-$. Ignoring for our purposes the reducing (Q) side of PS II, we may represent a photoact as: Chl $a_2 \rightarrow \text{Chl } a_2^+$, 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 $a_2^+ + Z \rightarrow \text{Chl } a_2 + Z^+$. $Z^+$, in turn, undergoes the $\text{HCO}_3^-$-mediated reaction: $Z^+ + S_n \rightarrow Z + S_{n+1}$, where $S$ is the charge accumulating enzyme or system in the $n$th state ($n = 0, 1, 2$) 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 $\text{HCO}_3^-$, we can imagine it occurs simply as: $Z^+ + S_1 \rightarrow S_1^*$; $S_1 + O_2$ precursor(s) $\rightarrow S_0 + O_2$.

A second possibility is that $Z^+$ and $S_1$ cooperate as: $Z^+ + S_0 + O_2$ precursor(s) $\rightarrow Z + S_0 + O_2$.

Thus, in the above model, $\text{HCO}_3^-$ controls the transfer of the first three electrons from the positive charge-accumulating mechanism to oxidized $Z$.

An alternative explanation of the $\text{HCO}_3^-$ 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 $a_2 \rightarrow \text{Chl } a_2^+$; Chl $a_2^+ + S_n \rightarrow \text{Chl } a_3 + S_{n+1}$, where $n$ is again equal to 0, 1, or 2. In this model $\text{HCO}_3^-$ 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 $a_3^+$.

Besides accelerating the relaxation reactions, $\text{HCO}_3^-$ 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 $\text{HCO}_3^-$, less time might be available for a back reaction of Chl $a_3^+$ 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 $\text{HCO}_3^-$. If this is the case, we might expect greater amounts of delayed light emission (reflecting more misses) from $\text{HCO}_3^-$-depleted systems in the msec time range after a flash.

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