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ACTION OF BICARBONATE ON PHOTOSYNTHETIC ELECTRON TRANSPORT IN THE PRESENCE OR ABSENCE OF INHIBITORY ANIONS

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INTRODUCTION

Bicarbonate (or CO_2) was shown by Warburg and Krippahl [1] to stimulate electron transport during the Hill reaction. This phenomenon has been referred to as the bicarbonate (HCO_3^-) effect. The electron transport chain can be dissected into a number of clearly defined partial reactions through the addition of specific inhibitors and electron donors and acceptors. By applying this approach (see e.g., [2,3]) the HCO_3^- effect has been shown to be associated with the acceptor side of Photosystem II (PS II):

$$H_2O \longrightarrow OEC \longrightarrow Z \longrightarrow P680 \longrightarrow Pheo \longrightarrow Q_A \longrightarrow Q_B \longrightarrow PQ \qquad (1)$$

The electron donor side of PS II contains the oxygen-evolving complex (OEC), and a bound plastoquinol "Z" that supplies electrons to the reaction center chlorophyll (Chl) a P680. The electron acceptor side contains a pheophytin (Pheo) molecule and two plastoquinones, Q_A and Q_B. Electrons are transferred from $Pheo^-$ to Q_A, which can only be reduced to the semiquinone form. After two such events, Q_B is reduced to plastoquinol. The plastoquinol, Q_BH_2, is then able to exchange with the plastoquinone (PQ) pool to provide a second Q_B molecule for subsequent reduction. This two-electron transfer step at the Q_B level is known as the two-electron gate (see e.g., [4]). Stoichiometrically, two PQH_2 molecules are formed and two water molecules oxidized for each O_2 evolved. The resultant PQH_2 molecules are oxidized at the plastoquinol-plastocyanin oxidoreductase or cytochrome b_6/f complex. The oxidation of H_2O and PQH_2 supply protons to the internal thylakoid space for chemiosmotic coupling.

The oxygen-evolving mechanism has been described by a kinetic model which recognizes 5 separate oxidation states or S-states [5]:

$$S_0 \rightleftharpoons S_0' \longrightarrow S_1 \rightleftharpoons S_1' \longrightarrow S_2 \rightleftharpoons S_2' \longrightarrow S_3 \rightleftharpoons S_3' \longrightarrow S_4 \xrightarrow[O_2]{} S_0 \qquad (2)$$

A single photoact advances an S-state from S_n to S_n' while the transition from S_n' to S_{n+1} represents a recovery reaction before a center is able to utilize a second photon. Molecular O_2 is released during the S_4 to S_0 transition. For a recent review of PS II the reader is referred to [6] and of the OEC to [7].

The Bicarbonate Effect

Although it has been suggested [8,9] that HCO_3^- plays a role on the water oxidation side of PS II, as yet no firm evidence is available to show a significant, large, and direct effect of HCO_3^- before P680. On the other hand, a role of HCO_3^- on the PQ reduction side of PS II, at least in the presence of HCO_2^-, has been firmly established (see reviews [10-13]).

Fig. 1 shows the scheme of electron flow from Q_A^- to the PQ pool via the two electron acceptor Q_B; also shown are the steps that are affected by HCO_2^-/HCO_3^-: the major effect of HCO_3^-, at least in the presence of HCO_2^-, is to facilitate the passage of electrons through the two-electron gate. The specific effect may involve mediation in the protonation reactions. The principal arguments to support the action of HCO_3^-, shown in Fig. 1, are: (1) the reoxidation of Q_A^-, as measured by the Chl *a* fluorescence yield decay [15,16] or by the absorbance change at 320 nm [17,18], is stimulated ten- to twenty-fold by HCO_3^- in HCO_3^--depleted membranes; (2) in PS II particles, a light- and chemically-induced EPR signal (g = 1.82), attributed to the Q_A^--Fe^{2+} complex, is reversibly increased in amplitude by a factor of about 10 by HCO_3^--depletion [19]; (3) The Chl *a* fluorescence yield [20] and thermoluminescence [21] after a series of single flashes of light suggest a dramatic slowing down of electron flow after the third and subsequent flashes following HCO_3^--depletion, which is totally reversed upon HCO_3^--addition; this suggests that there is a drastic HCO_3^- effect on the exchange of Q_B^{2-} with PQ at the Q_B-apoprotein and (4) HCO_3^--depletion causes a several-fold change in the affinity of the binding of ^{14}C-atrazine, which binds in the Q_B-apoprotein region [22; cf. 23]. In contrast, attempts to observe a HCO_3^- effect on (a) the OEC to Z^+ electron transfer kinetics, EPR signal II_{vf} [15]; (b) Z to $P680^+$ electron flow by the kinetics of the fast Chl *a* fluorescence rise [15]; (c) the H_2O to Q_A reaction with ferricyanide as an electron acceptor in trypsin-treated thylakoids [24]; and (d) the kinetics of O_2 evolution after the third flash [12], have failed thus far.

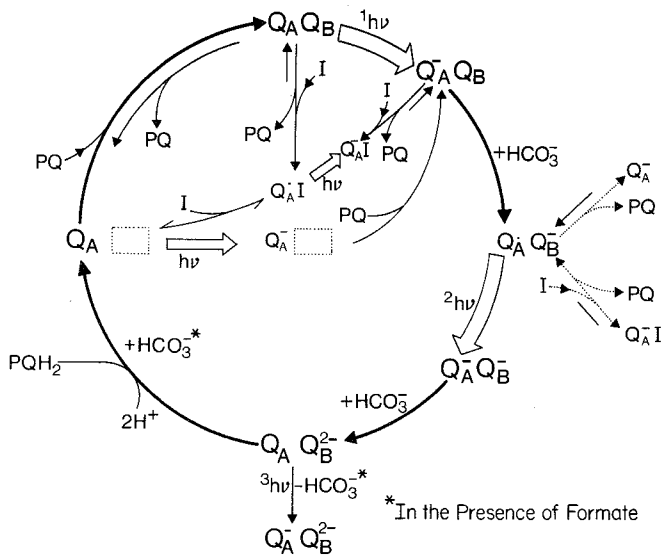

Fig. 1. A Scheme for the Effect of Bicarbonate in the Presence of Formate on the Electron Transfer on the Acceptor Side of Photosystem II. In this scheme, I stands for Inhibitor/herbicide, Q_A for the first bound-quinone electron acceptor, Q_B for the second bound-quinone electron acceptor, PQ for the plastoquinone pool, $^nh\nu$ for the n^{th} flash. (The scheme is self-explanatory, and is modified after the scheme presented earlier [13,14].)

Recently it has been reported that HCO_3^- is able to reverse the inhibition of PS II by a number of monovalent anions [25]. In addition to reversing the inhibition by formate, HCO_3^- was also effective when NO_2^-, NO_3^-, F^- and acetate were used as inhibitory anions. This work confirmed and extended the earlier findings of Good [26] by including NO_2^- and supports the notion that HCO_3^- is binding to a more general anion binding site [27]. All of these anions exhibit competitive inhibition of HCO_3^- binding to the anion binding site [25,27]. The binding constant of HCO_3^- has been determined and is approximately 0.08 mM [27]. Although it has been suggested [27-29] that some electron transport proceeds when the anion binding site is empty, only HCO_3^- facilitates electron transfer through the two-electron gate.

THE ACTIVE SPECIES

To determine whether CO_2 or HCO_3^- is the species required, advantage was taken of the pH dependence of the ratio $[CO_2]/[HCO_3^-]$ at equilibrium. This ratio increases with lower pH, because an increase in $[H^+]$ drives the following reaction toward the left:

$$CO_2 + H_2O \xleftarrow{K_1} H_2CO_3 \xleftarrow{K_2} H^+ + HCO_3^- \xleftarrow{K_3} 2H^+ + CO_3^{2-} \qquad (3)$$

K_1, K_2, and K_3 are known equilibrium constants ($K_1 = (1.4 \pm 0.2) \times 10^{-3}$; $K_2 = (3.2 \pm 0.4) \times 10^{-4}$M; $K_3 = 4.70 \times 10^{-11}$M [28]), from which the relative ratios of reactants to products can be calculated. When $[H^+]$ and the total concentration of all carbonate species are known, then $[CO_2]$ and $[HCO_3^-]$ at equilibrium can be calculated:

$$[HCO_3^-]_{eq} = \frac{[HCO_3^-]_i}{\frac{[H^+]}{K_1 \cdot K_2} + \frac{[H^+]}{K_2} + 1 + \frac{K_3}{[H^+]}} \qquad (4)$$

$$[CO_2]_{eq} = \frac{[H^+]}{K_1 \cdot K_2} [HCO_3^-]_{eq} \qquad (5)$$

The subscripts "eq" and "i" refer to equilibrium and initial conditions, respectively.

Spinach thylakoids were depleted of CO_2 to inhibit the Hill reaction, following a procedure similar to [31]. The percentage of control activity restored to these thylakoids was then measured as a function of the total $[HCO_3^-]$ that was added, and the experiment repeated at a variety of pHs. Figures 2 and 3 show the same data plotted against the equilibrium $[HCO_3^-]$ and against the equilibrium $[CO_2]$, respectively. When plotted against $[HCO_3^-]_{eq}$ there is no apparent pH dependence; even though the $[CO_2]/[HCO_3^-]$ ratio varies four-fold over the pH range, each curve falls on top of the others. This means that $[CO_2]_{eq}$ has no apparent effect on the degree of restoration. On the other hand, when plotted against $[CO_2]_{eq}$, the curves become steeper with increasing pH. From equation (5) it is obvious that the ratio $[HCO_3^-]_{eq}/[CO_2]_{eq}$ is constant at any given pH, but changes proportionately with any change in $[H^+]$. Therefore, at any given $[CO_2]_{eq}$, the $[HCO_3^-]_{eq}$ is greater at higher pH. Thus, the pH dependence of the curves in Fig. 3 and the lack of such dependence in Fig. 2 is evidence that HCO_3^-, not CO_2, is responsible for restoring the Hill reaction. The inset to Fig. 3 shows the effect of $[HCO_3^-]_{eq}$ on the Hill activity, with $[CO_2]_{eq}$ held constant. The $[HCO_3^-]_{eq}$ was calculated from equation (5), and the percent

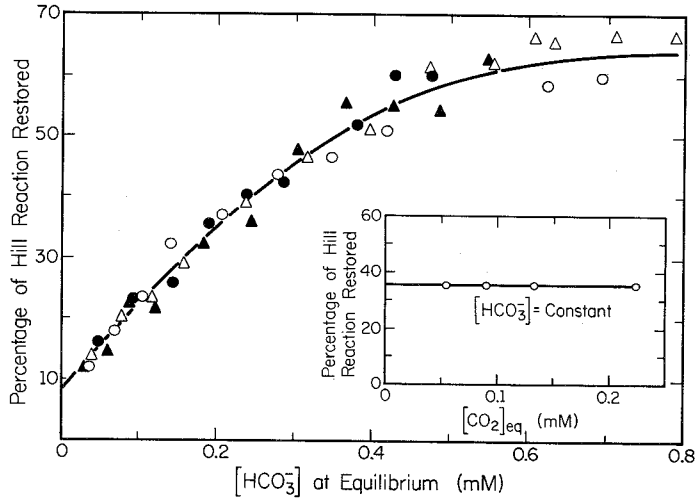

Fig. 2. The Percentage of Control Activity Restored to CO_2-depleted Spinach Thylakoids as a Function of the Equilibrium $[HCO_3^-]$. The Hill activity was measured by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) as the decrease in absorbance at 600 nm. Illumination began 3 min after the addition of $NaHCO_3$. Symbols: closed circles, pH 6.30; closed triangles, pH 6.53; open circles, pH 6.7; open triangles, pH 6.91. Inset: The effect of $[CO_2]_{eq}$ on the Hill activity, with $[HCO_3^-]$ held constant at 0.2 mM, taken from the curves shown in the main portion of the figure (Blubaugh and Govindjee, 1984, unpublished)

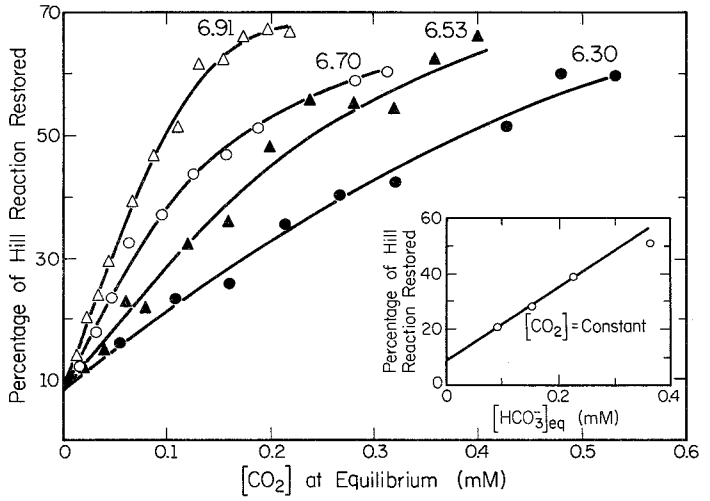

Fig. 3. The Percentage of Control Activity Restored to CO_2-depleted Spinach Thylakoids as a Function of the Equilibrium $[CO_2]$. The symbols and protocol are the same as in Fig. 2. Inset: The effect of $[HCO_3^-]_{eq}$ on the Hill activity, with $[CO_2]_{eq}$ held constant at 0.1 mM, taken from the curves in the main portion of the figure (Blubaugh and Govindjee, 1984, unpublished).

control activity was taken from the curves of Fig. 3 at the point on each curve where $[CO_2]_{eq}$ = 0.1 mM. Following the same method, the inset to Fig. 2 shows the effect of $[CO_2]_{eq}$ on the Hill activity with $[HCO_3^-]_{eq}$ held constant at 0.2 mM. It is clear that the effect of HCO_3^- is independent of $[CO_2]_{eq}$.

In the analysis thus far, no consideration has been given to the possibility of H_2CO_3 or CO_3^{2-} as the active species, primarily because both exist at extremely low concentrations in the system described here. However, both species can be ruled out from the data presented. The $[H_2CO_3]_{eq}/[CO_2]_{eq}$ ratio is equal to K_1 (1.4×10^{-3}) and is independent of pH. Thus, a plot of activity versus $[H_2CO_3]_{eq}$ would qualitatively appear identical to the data in Fig. 3. Since $[H_2CO_3]_{eq}$ is directly proportional to $[CO_2]_{eq}$ at all pHs, identical arguments can be made for H_2CO_3 as were made for CO_2. Thus, H_2CO_3 cannot be the required species. CO_3^{2-} exists at only 10^{-3}-10^{-4} times the concentration of HCO_3^- at the pHs used here. The ratio of $[CO_3^{2-}]_{eq}$ to $[HCO_3^-]_{eq}$ is equal to $K_3/[H^+]$. Since this ratio is inversely proportional to $[H^+]$, the curves in Fig. 2 would be expected to show a pH dependence if CO_3^{2-} were involved in restoration of the Hill activity. As was the case with CO_2, the lack of such pH dependence in Fig. 2 suggests that CO_3^{2-} is not involved. Only if the system was not at equilibrium at the time of each measurement could the curves in Fig. 2 show no apparent pH dependence if CO_3^{2-} was involved. However, from a time course of the restoration after addition of a half-saturating $[HCO_3^-]$, we have concluded that equilibrium is reached in 2-2.5 min (data not shown). Since the measurements in Figs. 2 and 3 were made 3 min after HCO_3^- addition, CO_3^{2-} can be ruled out as having any involvement.

The possibility that CO_2 (with perhaps some contribution from H_2CO_3) is required for diffusion to the active site is not disputed by the data presented here. These measurements were made under equilibrium conditions and do not reflect the kinetics of bicarbonate binding. Some experiments, done under non-equilibrium conditions, indicate that CO_2 is required. For example, when HCO_3^- is added to HCO_3^--depleted thylakoids, a lag is observed before the Hill reaction is stimulated, whereas CO_2 stimulates the Hill reaction sooner [32,33]. Presumably the initial $[CO_2]$ determines how quickly bicarbonate is able to reach the binding site. Similarly, from the effect of pH on the rate of HCO_3^- binding, it was suggested that CO_2 is required [34]. While the equilibrium $[HCO_3^-]$ in the vicinity of the binding site is shown here to be a critical factor, the $[CO_2]$ could be an important kinetic consideration if the binding site is buried beneath a hydrophobic domain.

THE ANION BINDING SITE

Recently the possibility has been raised [27-29] that the stimulatory HCO_3^- effect is a simple reversal of the inhibitory HCO_2^- effect [26]. Snel et al. [28] and Snel and van Rensen [29] entertain the possibility that both the HCO_2^- and the HCO_3^- effects may be of physiological significance, since formate is produced in peroxisomes. The binding constant for HCO_3^-, calculated by these authors, is about 0.08 mM, whereas the $[CO_2]$ in photosynthesizing chloroplasts is estimated to be only 0.005 mM [35]. Thus, one may argue that under normal conditions, all binding sites are empty, and there may not be any real role for HCO_3^- *in vivo*. However, since it is $[HCO_3^-]$ and not $[CO_2]$ which determines the degree of functioning, there is no good reason to assume that the sites are empty. For example, if, in the vicinity of the binding site, the pH is 8, then the $[HCO_3^-]$ in equilibrium with 0.005 mM CO_2 is 0.22 mM, well above the binding constant. The location of the HCO_3^- binding site is, therefore, an important question to answer.

It is also possible that the binding constant has been overestimated, because of the salt concentration at which it was determined. This possibility is discussed later. The key question of whether HCO_3^- is required

for electron flow in a normal system needs to be tested. Experiments must be done with totally HCO_3^--depleted samples, with the HCO_3^- concentration measured, since a tightly bound HCO_3^- may have been present in many experiments. Furthermore, experiments should be done to see if the HCO_3^- effect can be obtained without the use of HCO_2^-; this is a definite way to test if HCO_3^- stimulation is a reversal of the HCO_2^- effect.

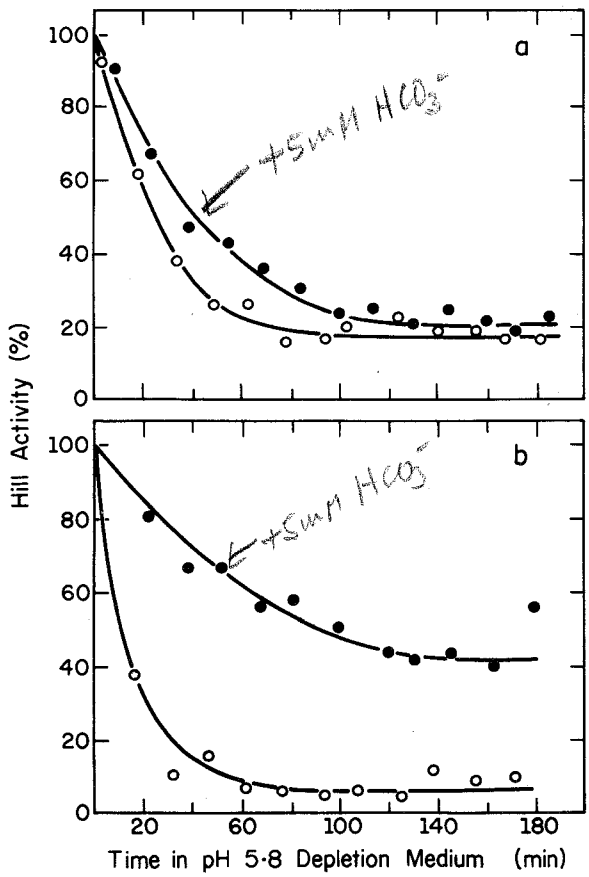

Fig. 4. The Percentage of Hill Activity Remaining after Incubation in a CO_2-free HCO_3^--depletion Medium at pH 5.8. The depletion medium contained 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and 10 mM sodium phosphate (pH 5.8). The Hill activity was assayed in a reaction medium containing 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and 25 mM sodium phosphate (pH 6.5). 25 mM HCO_2^- was also present in both the depletion and reaction media in (b). The spinach thylakoids used in this experiment had been stored in liquid N_2. The electron acceptor was methyl viologen. For other experimental details, see [3]. In (a) 100% = 896 μequiv./mg Chl/hr and in (b) 100% = 584 μequiv./mg Chl/hr (Eaton-Rye and Govindjee, 1985, unpublished)

The Bicarbonate Effect in the Absence of Formate

We have already shown [3] the existence of a stimulatory effect of HCO_3^- on Q_A^- to Q_B electron flow, when HCO_2^- was omitted from both the depletion medium and the reaction medium. This determination was made by following the decay of the Chl a variable fluorescence. As was observed in formate-containing membranes [16,20], maximal inhibition occurred after the third actinic flash. However, in the absence of formate, the extent of this inhibition was decreased ($t_{1/2}$ for HCO_3^--depleted in the presence of HCO_2^-, ~ 15 ms; for HCO_3^--depleted in the absence of HCO_2^-, ~ 2.5 ms; for HCO_3^--resupplied and control, ~ 0.35 ms.). In the formate-free case we found that after approximately a 60 min depletion the half-time of Q_A^- reoxidation did continue to increase but the effect became increasingly irreversible. Fig. 4 demonstrates a similar observation in the Hill reaction. Thus it appears, under these conditions, that if the anion binding site is empty, irreversible

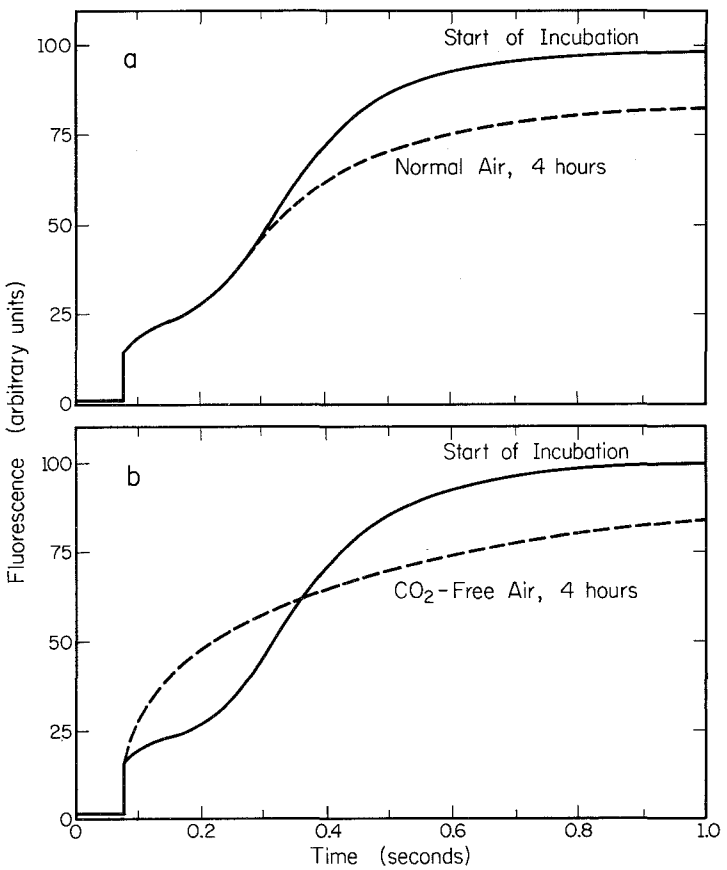

Fig. 5. The Effect of CO_2-depletion, in the Absence of HCO_2^-, on the Variable Chl a Fluorescence Transient. (a) The fluorescence transient at the start of incubation and after 4 hr of incubation under a stream of normal air. (b) The fluorescence transient at the start of incubation and after 4 hr of incubation under a stream of CO_2-free air. The area above the curve for the CO_2-depleted sample is approximately 60% of that for the non-CO_2-depleted sample. Spinach thylakoids were suspended in 50 mM phosphate, pH 6.4, 15 mM NaCl, and 5 mM $MgCl_2$ (Blubaugh and Govindjee, 1985, unpublished).

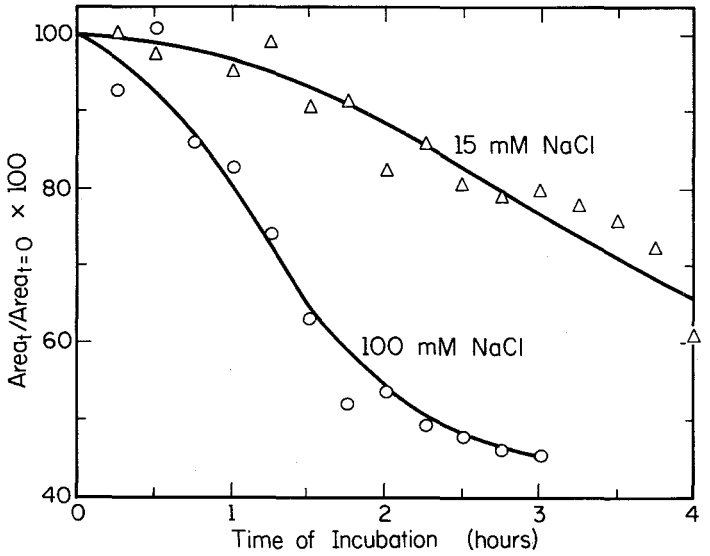

Fig. 6. The Time Course of CO_2-depletion in the Absence of HCO_2^-, by Incubation under CO_2-free air, under Low and High Salt Concentrations. The area above the fluorescence transient is plotted as a function of time under CO_2-free air. Area $_{t = 0}$ is the area above the transient at the start of the incubation, and Area$_t$ is the area at the time indicated. Symbols: circles, 100 mM NaCl; triangles, 15 mM NaCl (Blubaugh and Govindjee, 1985, unpublished).

inactivation of PS II occurs. Fig. 4b shows that the presence of 25 mM formate actually acts in a protective role. In addition, raising the pH to 6.5 also prevented the irreversible loss of activity shown in Fig. 4a. A two-fold HCO_3^- effect was observed under the same experimental conditions as in Fig. 4a after 120 min in the depletion medium at pH 6.5. The electron transport rates (H_2O to methylviologen (MV)) were: for HCO_3^--depleted, 162 μequiv./mg Chl/hr; for HCO_3^--resupplied, 368 μequiv./mg Chl/hr.

Similarly, merely passing CO_2-free air over a formate-free sample of thylakoids at pH 6.4 is enough to alter the kinetics of the variable Chl *a* fluorescence (Fig. 5b) in a manner similar to HCO_3^--depletion. Passing normal air over an identical sample had no such effect (Fig. 5a). The level of maximum fluorescence was equally diminished for both samples; this effect was probably due to aging of the thylakoids. Fig. 6 shows the time course of this effect for spinach thylakoids suspended at two different salt concentrations, determined from the relative areas over the curves. It is apparent that HCO_3^- is removed more readily at higher [NaCl]. This could be due to ionic strength effects on the HCO_3^- binding site, or to the charge density at the membrane surface, which would alter the pH in the vicinity of the membrane. The value of 0.08 mM, mentioned earlier, for the binding constant of HCO_3^- had been determined at a salt concentration of 200 mM [25]. Since at lower [NaCl] the HCO_3^- appears to be held more tightly, the binding constant may be much lower at physiological ionic strength.

Formate has been shown to be competitive with the HCO_3^- binding site [16,27,36]. This conclusion can now be extended to a number of anions which inhibit the Hill reaction and which also increase the dissociation constant for HCO_3^-. The most effective anion that has been tested is nitrite (NO_2^-) [25]. We have repeated the experiment shown in Fig. 4b, but we replaced the

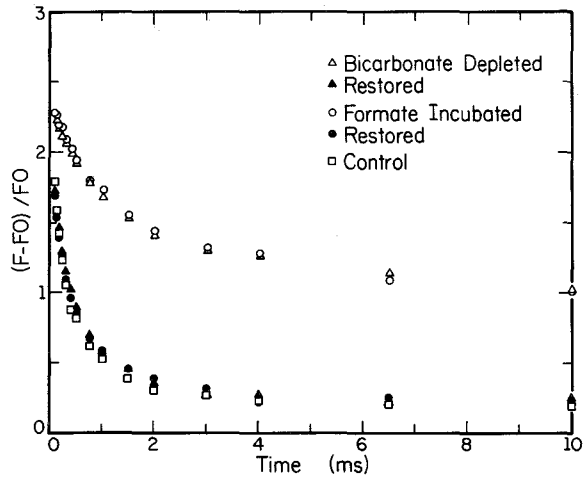

Fig. 7. The Decay of the Variable Chl a Fluorescence after Three Actinic Flashes Spaced at 1 s. FO is the Chl a fluorescencae yield from the measuring flash when all Q_A was oxidized and F was the yield at the indicated time after the actinic flash. The reaction medium (see Fig. 4) was supplemented with 25 mM HCO_2^-, 0.1 mM methyl viologen and 0.1 mM gramicidin. Pea thylakoids were used and the half-times were determined as in [16] (see text). The figure is redrawn from [16].

25 mM HCO_2^- with 25 mM NO_2^-. Our results indicate that nitrite behaves in a similar way to formate and prevents the irreversible loss of activity shown in Fig. 4a. A 7-fold HCO_3^- effect was observed in the presence of 25 mM NO_2^- after 30 min in the depletion medium at pH 5.8. The electron transport rates (H_2O to MV) were: for HCO_3^--depleted, 75 μequiv./mg Chl/hr; for HCO_3^--resupplied, 539 μequiv./mg/ Chl/hr.

The Relationship Between the Anion Binding Site and the Acceptor Side Bicarbonate Effect

Data are presented in Fig. 7 from an earlier study [16], in which the effect of HCO_3^--depletion in the presence of HCO_2^- and the effect of HCO_2^- incubation in the presence of atmospheric CO_2 were compared. The decay of the variable Chl a fluorescence after one or three actinic flashes was nearly identical with either HCO_2^--incubated or HCO_3^--depleted samples. With both preparations, the addition of 10 mM HCO_3^- restored the decays to the pattern seen with control thylakoids. Twenty-five mM HCO_2^- was present in these experiments. A similar observation has been made for the Hill reaction [37], but in this instance 100 mM HCO_2^- was required to observe a HCO_3^--reversible inhibition in HCO_2^--incubated samples [cf. 16]. The results in Fig. 7 suggest that HCO_2^- and HCO_3^- compete for a site on the reducing side of PS II. It is evident that if HCO_2^- is present at a low HCO_3^- concentration, forward electron flow is slowed; if HCO_3^- is present in excess, or at physiological concentrations in the absence of formate, the normal flow is restored. It was also reported in [16] that during HCO_2^--incubation the transition from a fast to a slow decay of the variable Chl a fluorescence was accelerated under any of the following conditions: a) the concentration of

HCO_2^- was increased; b) the pH was lowered; or c) when the CO_2 concentration was lowered. These observations all support the hypothesis that HCO_2^- and HCO_3^- compete for a site on the reducing side of PS II, and they further support the conclusion that HCO_3^-, not CO_2, is the active species.

In a recent study of the Chl *a* fluorescence transient, Blubaugh and Govindjee [38] found that addition of excess HCO_3^- to non-depleted thylakoids resulted in an enhanced rate of Chl *a* fluorescence rise in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This result indicated that addition of excess HCO_3^- to these thylakoids either inhibits a back-reaction with the donor side or prevents some reoxidation of Q_A^- by auxilliary acceptors of PS II (see *e.g.*, [39]). It has also been shown that addition of excess HCO_3^- to non-depleted thylakoids inhibits the Hill reaction in the presence of silicomolybdate (SiMo) [40-42], which is thought to accept electrons at the level of Q_A. This result and the work of Blubaugh and Govindjee [38] suggest that two HCO_3^- binding sites exist. We therefore asked if the HCO_3^- binding site that gave rise to a dissociation constant of 0.08 mM for HCO_3^- was the specific site for HCO_3^- that facilitates electron transfer through the two-electron gate. Since the most effective competitor of HCO_3^- binding so far studied is NO_2^- [25], we studied the effect of NO_2^- incubation on Q_A^- reoxidation. As in the presence of HCO_2^-, we observed a maximum inhibition after the third actinic flash (Fig. 8). The half-time for the NO_2^--incubated sample was ~ 15 ms, which is nearly identical to the result obtained from the data of Fig. 7. A similar result was obtained for NO_2^--incubated samples in the presence of 10 mM hydroxylamine (HA) (data not shown), as was earlier shown with HCO_2^- in the presence of HA [3]. Therefore, our data strongly support the hypothesis that the anion binding site is the site where HCO_3^- binds and facilitates electron transfer through the two-electron gate.

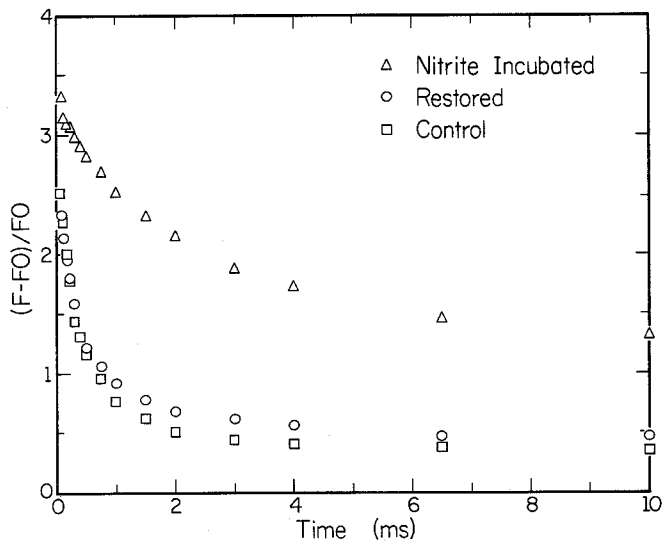

Fig. 8. The Decay of the Variable Chl *a* Fluorescence after Three Actinic Flashes Spaced at 1 s. Spinach thylakoids were used. Other conditions were as in Fig. 7 except that 25 mM HCO_2^- was replaced by 25 mM NO_2^- (Eaton-Rye and Govindjee, 1985, unpublished).

THE ACTION OF BICARBONATE AT THE LEVEL OF THE TWO-ELECTRON GATE

The operation of the two-electron gate of PS II, neglecting the precise nature of the protolytic steps, is summarized in equations 6-10:

$$Q_AQ_B \Longrightarrow Q_A^-Q_B \longleftrightarrow Q_AQ_B^- \quad (6)$$

$$Q_AQ_B^- \Longrightarrow Q_A^-Q_B^- \longleftrightarrow Q_AQ_B^{2-} \quad (7)$$

$$Q_AQ_B^{2-} \longleftrightarrow Q_A + PQH_2 \quad (8)$$

$$Q_A + PQ \longleftrightarrow Q_AQ_B \quad (9)$$

$$Q_AQ_B \Longrightarrow Q_A^-Q_B \longleftrightarrow Q_AQ_B^- \quad (10)$$

Following a short, single-turnover actinic flash given to dark-adapted thylakoids, an electron is placed on Q_A. This electron is then transferred to Q_B with a half-time of ~ 0.15 ms (see e.g., [43]; Eq. 6). The equilibrium established for the sharing of an electron between Q_A and Q_B greatly favors centers in the state $Q_AQ_B^-$ [43]; these centers are open and able to undergo a second photoact (Eq. 7). The second electron is transferred to Q_B^- with a half-time of ~ 0.3 ms (see e.g., [43]). The resulting plastoquinol is then able to exchange with a plastoquinone from the PQ pool at the Q_B binding site (Eqs. 8 and 9). If a dark-time of sufficient duration is given before a third actinic flash, the kinetics of electron transfer from Q_A^- to Q_B in equation 10 will resemble those obtained after the first flash (Eq. 6). The minimum dark-time between flash 2 and flash 3 where the kinetics of Q_A^- reoxidation resemble those observed after the first actinic flash is defined here as the turnover time of the two-electron gate. This measurement has recently been made by H.H. Robinson and A.R. Crofts (personal communication) and found to have a half-time of < 2.5 ms in control thylakoids.

We have varied the dark-time between the second and third actinic flashes in HCO_3^--depleted (with HCO_2^- present) and HCO_2^--incubated thylakoids. The dark-times were: 30 ms, 50 ms, 100 ms, 250 ms, 500 ms, and 1 s. In each case maximal inhibition of Q_A^- reoxidation occurred after the third flash and exhibited a $t_{1/2}$ of ~ 15 ms (data not shown). These findings suggest that even after 1 s, centers that have been depleted of HCO_3^- or inhibited by HCO_2^- are unable to exchange with the PQ pool at their Q_B binding site. In each case the effect is fully reversible and control kinetics are completely restored upon the addition of HCO_3^-.

The rate of Q_A^- reoxidation after the third actinic flash in HCO_3^--depleted samples is identical to all subsequent actinic flashes (Fig. 9, also see [16,20]). The same result is obtained with both HCO_2^-- and NO_2^--incubation (data not shown). The decay kinetics after the second flash are intermediate between those following flash 1 and 3 (see [16]).

A possible explanation for these observations is that the binding of inhibitory anions to PS II may alter the association constant, K_0, for Q_B. From equation (9):

$$K_0 = [Q_A \cdot Q_B]/([Q_A] + [PQ_{(pool)}]) \quad (11)$$

Although there is no direct measure of the value for K_0, a number of methods for estimating a value are available [44]. One method is to analyze the decay kinetics of Q_A^- by monitoring the variable Chl a fluorescence after a single flash. Biphasic kinetics are observed for this decay; 60-70% of centers undergo oxidation by a first-order process with a half-time of ~ 0.15 ms and the remainder by slower processes of indeterminate order [44]. If it is assumed that the centers exhibiting first-order kinetics represent centers

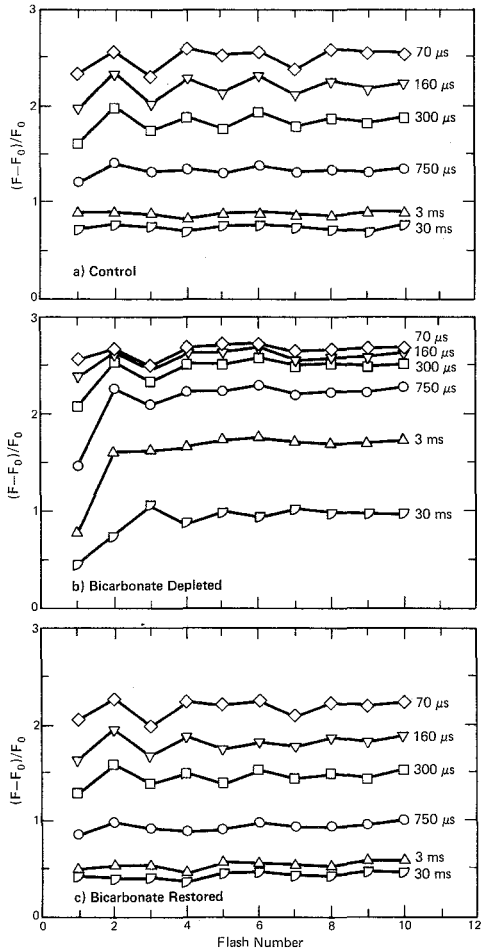

Fig. 9. The Effect of HCO_3^--depletion, in the Presence of 25 mM HCO_2^-, on the Fluorescence Flash Pattern. The times indicated are when the measuring flash was fired. Spinach thylakoids were used; other details are as in Fig. 7 (Eaton-Rye and Govindjee, 1984, unpublished)

in the state Q_AQ_B before the flash, a value of 500 M^{-1} for K_0 can be calculated [44]. We have analyzed our earlier data for HCO_3^--depleted and HCO_2^--incubated thylakoids [16] and found that K_0 is reduced to 200 M^{-1} in these samples. A second effect is also evident from this analysis. The half-time of the fast phase is increased approximately 3-fold (i.e., from ~ 0.2 ms to ~ 0.6 ms) in these samples [cf. 16]. The mechanism of this second effect cannot be explained from the available data.

In addition to the slowing and reduction of the fast phase of Q_A^- reoxidation, a shift in the equilibrium for the sharing of an electron between Q_A and Q_B (see Eq. 6) has been reported in thylakoids that have been HCO_3^--depleted in the presence of formate [45]. A two-fold shift in this equilibrium towards Q_A^- was observed by comparing the rates of the back-reaction with S_2 both in the presence and absence of DCMU. In the absence of DCMU, the back-reaction from Q_B^- to S_2 was unaffected by HCO_3^--depletion, but

in the presence of DCMU the back-reaction of Q_A^- with S_2 was inhibited two-fold [45].

The equilibrium for the sharing of an electron between Q_A and Q_B is pH dependent [46]. It has therefore been suggested that the presence of a proton in association with the Q_B site stabilizes the electron on $Q_B^-(H^+)$. A possible explanation for the observed shift in this equilibrium may be that HCO_3^--depletion inhibits protonation at the Q_B site. In addition, the fraction of centers decaying through the rapid first-order process has been shown to be proportional to the fraction of centers in which $Q_B^-(H^+)$ is present before the second flash [46]. Therefore the inhibition on the $Q_B^-(H^+)$ protonation suggested above may also account for the inhibited kinetics of Q_A^- reoxidation observed after the second flash in HCO_3^--depleted membranes [16,20]. By analogy, the maximal inhibition observed after the third flash may result from Q_B^{2-} not becoming protonated and therefore not able to exchange with the PQ pool. This interpretation suggests that the rate-limiting step introduced by HCO_3^--depletion and/or anion inhibition is the rate of protonation of Q_B^{2-}.

Recently the ability of carbonic anhydrase inhibitors to inhibit PS II has been reported [25,42]. This approach to the bicarbonate problem has also led to the suggestion that HCO_3^- functions as a proton donor/acceptor at the anion binding site [25,47]. These workers have also reported an inhibitory effect of HCO_3^- on PS II at pH 8.0 [34,42]. However, the reoxidation of Q_A^- was unaffected by the addition of HCO_3^- in these samples [42]. On the other hand, a slowing of the $S_2' \longrightarrow S_3$ transition was shown, but this phenomenon appears to be distinct from the HCO_3^- effect under consideration here. Addition of 10 mM HCO_3^- to non-depleted thylakoid membranes at pH 6.0 and 7.0 did not inhibit the Hill reaction supported by $K_3Fe(CN)_6$ or MV [48].

The inhibition of the back-reaction of Q_A^- with S_2 in the presence of DCMU [45] presents other interesting possibilities arising as a consequence of HCO_3^--depletion. The apparent increase in the stability of Q_A^- is not reflected in a shift in the mid-point potential of the Q_A/Q_A^- couple [49]. Therefore the observed inhibition of the back-reaction between Q_A^- and S_2 in HCO_3^--depleted thylakoids may indicate an additional effect of HCO_3^--depletion and/or anion incubation on the donor side of PS II . However, this result may also be explained by HCO_3^--depletion altering the redox potential of the Fe^{3+}/Fe^{2+} couple associated with PS II. This interpretation may also explain the increased EPR signal (g = 1.82) attributed to Q_A^--Fe^{2+} observed in PS II particles [19].

CONCLUSIONS

Our findings here support the following conclusions: (1) the active species of the bicarbonate effect is HCO_3^-, not CO_2 or CO_3^{2-}; (2) NO_2^- (as is the case with HCO_2^-) inhibits the electron acceptor side of PS II; this inhibition is also reversed by HCO_3^-; (3) both HCO_2^- and NO_2^- protect PS II against irreversible damage at pH 5.8; and (4) in the complete absence of inhibitory anions, HCO_3^--depletion still inhibits the electron acceptor side of PS II. Furthermore, we suggest that the unique ability of HCO_3^- to stimulate electron transport may arise from its ability to participate in the protolytic reactions of the two-electron gate.

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REFERENCES

1. O. Warburg and G. Krippahl, Notwendigkeit der Kohlensäure für die Chinon-und Ferricyanid-Reaktionen in grünen Grana, Z. Naturforsch. 15B:367 (1960).
2. R. Khanna, Govindjee and T. Wydrzynski, Site of Bicarbonate Effect in Hill Reaction: Evidence from the Use of Artificial Electron Acceptors and Donors, Biochim. Biophys. Acta 462:208 (1977).
3. J. J. Eaton-Rye and Govindjee, A Study of the Specific Effect of Bicarbonate on Photosynthetic Electron Transport in the Presence of Methyl Viologen, Photobiochem. Photobiophys. 8:279 (1984).
4. B. R. Velthuys, Electron-dependent Competition Between Plastoquinone and Inhibitors for Binding to Photosystem II, FEBS Lett. 126:277 (1981).
5. B. Kok, B. Forbush and M. McGloin, Cooperation of Charges in Photosynthetic O_2 Evolution-1: A Linear Four Step Mechanism, Photochem. Photobiol. 11:457 (1970).
6. H. J. van Gorkom, Electron Transfer in Photosystem II, Photosynth. Res. 6:97 (1985).
7. Govindjee, T. Kambara and W. Coleman, The Electron Donor Side of Photosystem II: The Oxygen Evolving Complex, Photobiochem. Photobiol. 40: in the press (1985).
8. H. Metzner, K. Fischer and O. Bazlen, Isotope Ratios in Photosynthetic Oxygen, Biochim. Biophys. Acta 548:287 (1979).
9. A. Stemler, Inhibition of Photosystem II by Formate: Possible Evidence for a Direct Role of Bicarbonate in Photosynthetic Oxygen Evolution, Biochim. Biophys. Acta 593:103 (1980).
10. Govindjee and J.J.S. van Rensen, Bicarbonate Effects on the Electron Flow in Isolated Broken Chloroplasts, Biochim. Biophys. Acta 505:183 (1978).
11. W. F. J. Vermaas and Govindjee, Unique Role(s) of Carbon Dioxide and Bicarbonate in the Photosynthetic Electron Transport System, Proc. Indian Natl. Sci. Acad. B47:581 (1981).
12. W. F. J. Vermaas and Govindjee, Bicarbonate or Carbon Dioxide as a Requirement for Efficient Electron Transport on the Acceptor Side of Photosystem II, in: "Photosynthesis Vol. II: Development, Carbon Metabolism and Plant Productivity," Govindjee, ed., Academic Press, New York (1983).
13. Govindjee, I. C. Baianu, C. Critchley and H. S. Gutowsky, Comments on the Possible Roles of Bicarbonate and Chloride Ions in Photosystem II, in: "The Oxygen Evolving System of Photosynthesis," Y. Inoue, A. R. Crofts, Govindjee, N. Murata, G. Renger, and K. Satoh, eds., Academic Press, Tokyo (1983).
14. Govindjee, The Oxygen Evolving System of Photosynthesis, in: "Advances in Photosynthesis Research Vol. I," C. Sybesma, ed., Martinus Nijhoff/Dr. W. Junk Publishers, The Hague (1984).
15. P. Jursinic, J. Warden and Govindjee, A Major Site of Bicarbonate Effect in System II Reaction: Evidence from ESR Signal II_{vf}, Fast Fluorescence Yield Changes and Delayed Light Emission, Biochim. Biophys. Acta 440:322 (1976).
16. H. H. Robinson, J. J. Eaton-Rye, J. J. S. van Rensen, and Govindjee, The Effects of Bicarbonate Depletion and Formate Incubation on the Kinetics of Oxidation-Reduction Reactions of the Photosystem II Quinone Acceptor Complex, Z. Naturforsch. 39c:382 (1984).
17. U. Siggel, R. Khanna, G. Renger and Govindjee, Investigation of the Absorption Changes of the Plastoquinone System in Broken Chloroplasts: The Effect of Bicarbonate Depletion, Biochim. Biophys. Acta 462:196 (1977).
18. J. Farineau and P. Mathis, Effects of Bicarbonate on Electron Transfer Between Plastoquinones in Photosystem II, in: "The Oxygen Evolving System of Photosynthesis," Y. Inoue, A. R. Crofts, Govindjee, N. Murata, G. Renger, and K. Satoh, eds., Academic Press, Tokyo (1983).

19. W. F. J. Vermaas and A. W. Rutherford, EPR Measurements on the Effects of Bicarbonate and Triazine Resistance on the Acceptor Side of Photosystem II, FEBS Lett. 175:243 (1984).
20. Govindjee, M. P. J. Pulles, R. Govindjee, H. J. van Gorkom and L. N. M. Duysens, Inhibition of the Reoxidation of the Secondary Electron Acceptor of Photosystem II by Bicarbonate Depletion, Biochim. Biophys. Acta 449:602 (1976).
21. Govindjee, H. Y. Nakatani, A. W. Rutherford and Y. Inoue, Evidence from Thermoluminescence for Bicarbonate Action on the Recombination Reactions Involving the Secondary Quinone Electron Acceptor of Photosystem II, Biochim. Biophys. Acta 766:416 (1984).
22. R. Khanna, K. Pfister, A. Keresztes, J. J. S. van Rensen and Govindjee, Evidence for a Close Spatial Location of the Binding Sites for CO_2 and for Photosystem II Inhibitors, Biochim. Biophys. Acta 634:105 (1981).
23. W. F. J. Vermaas, J. J. S. van Rensen, and Govindjee, The Interaction Between Bicarbonate and the Herbicide Ioxynil in the Thylakoid Membrane and the Effects of Amino Acid Modification on Bicarbonate Action, Biochim. Biophys. Acta 681:242 (1982).
24. J. J. S. van Rensen and W. F. J. Vermaas, Action of Bicarbonate and Photosystem II Inhibiting Herbicides on Electron Transport in Pea Grana and in Thylakoids of a Blue-Green Alga, Physiol. Plant. 51:106 (1981).
25. A. Stemler and J. B. Murphy, Bicarbonate: Reversible and Irreversible Inhibition of Photosystem II by Monovalent Anions, Plant Physiol. 77:974 (1985).
26. N. E. Good, Carbon Dioxide and the Hill Reaction, Plant Physiol. 38:298 (1963).
27. A. Stemler and J. B. Murphy, Determination of the Binding Constant of $H^{14}CO_3^-$ to the Photosystem II Complex in Maize Chloroplasts: Effects of Inhibitors and Light, Photochem. Photobiol. 38:701 (1983).
28. J. F. H. Snel, A. Groote-Schaarsberg and J. J. S. van Rensen, Mechanism and Physiological Role of Bicarbonate Action on Electron Flow: A Possible Link Between Photorespiration and Photosynthetic Electron Flow, in: "Advances in Photosynthesis Research Vol. I.", C. Sybesma, ed., Martinus Nijhoff/Dr. W. Junk Publishers, The Hague (1984).
29. J. F. H. Snel and J. J. S. van Rensen, Reevaluation of the Role of Bicarbonate and Formate in the Regulation of Photosynthetic Electron Flow in Broken Chloroplasts, Plant Physiol. 75:146 (1984).
30. W. Knoche, Chemical Reactions of CO_2 in Water, in: "Biophysics and Physiology of Carbon Dioxide," C. Bauer, G. Gros and H. Bartels, eds., Springer Verlag, New York (1980).
31. W. F. J. Vermaas and J. J. S. van Rensen, Mechanism of Bicarbonate Action on Photosynthetic Electron Transport in Broken Chloroplasts, Biochim. Biophys. Acta 636:168 (1981).
32. G. Sarojini and Govindjee, On the Active Species in Bicarbonate Stimulation of Hill Reaction in Thylakoid Membranes, Biochim. Biophys. Acta 634:340 (1981).
33. G. Sarojini and Govindjee, Is CO_2 An Active Species in Stimulating the Hill Reaction in Thylakoid Membranes?, in: "Photosynthesis II. Electron Transport and Photophosphorylation," G. Akoyunoglou, ed., Balaban International Science Services, Philadelphia, Pa. (1981).
34. A. Stemler, Forms of Dissolved Carbon Dioxide Required for Photosystem II Activity in Chloroplast Membranes, Plant Physiol. 65:1160 (1980).
35. J. D. Hesketh, J. T. Wooley and D. B. Peters, Predicting Photosynthesis, in: "Photosynthesis Vol. II.: Development, Carbon Metabolism and Plant Productivity," Govindjee, ed., Academic Press, New York (1983).
36. J. F. H. Snel and J. J. S. van Rensen, Kinetics of the Reactivation of the Hill Reaction in CO_2-Depleted Chloroplasts by Addition of Bicarbonate in the Absence and in the Presence of Herbicides, Physiol. Plant. 57:422 (1983).

37. J. F. H. Snel, D. Naber and J. J. S. van Rensen, Formate as an Inhibitor of Photosynthetic Electron Flow, Z. Naturforsch. 39c:386 (1984).
38. D. J. Blubaugh and Govindjee, Comparison of Bicarbonate Effects on the Variable Chlorophyll a Fluorescence of CO_2-Depleted and Non-CO_2-Depleted Thylakoids in the Presence of Diuron, Z. Naturforsch. 39c:378 (1984).
39. W. F. J. Vermaas and Govindjee, The Acceptor Side of Photosystem II in Photosynthesis, Photochem. Photobiol. 34:775 (1981).
40. R. Barr and F. L. Crane, Control of Photosynthesis by CO_2: Evidence for a Bicarbonate-Inhibited Redox Feedback in Photosystem II, Proc. Indiana Acad. Sci. 85:120 (1976).
41. F. L. Crane and R. Barr, Stimulation of Photosynthesis by Carbonyl Compounds and Chelators, Biochem. Biophys. Res. Comm. 74:1362 (1977).
42. A. Stemler and P. Jursinic, The Effects of Carbonic Anhydrase Inhibitors Formate, Bicarbonate, Acetazolamide, and Imidazole on Photosystem II in Maize Chloroplasts, Archiv. Biochem. Biophys. 221:227 (1983).
43. H. H. Robinson and A. R. Crofts, Kinetics of the Oxidation-Reduction Reactions of the Photosystem II Quinone Acceptor Complex, and the Pathway for Deactivation, FEBS Lett. 153:221 (1983).
44. A. R. Crofts, H. H. Robinson and M. Snozzi, Reactions of Quinones at Catalytic Sites: A Diffusional Role in H-Transfer, in: "Advances in Photosynthesis Research Vol. I.," C. Sybesma, ed., Martinus Nijhoff/Dr. W. Junk Publishers, The Hague (1984).
45. W. F. J. Vermaas, G. Renger and G. Dohnt, The Reduction of the Oxygen-Evolving System in Chloroplasts by Thylakoid Components, Biochim. Biophys. Acta 764:194 (1984).
46. H. H. Robinson and A. R. Crofts, Kinetics of Proton Uptake and the Oxidation-Reduction Reactions of the Quinone Acceptor Complex of PS II from Pea Chloroplasts, in: "Advances in Photosynthesis Research Vol. I.," C. Sybesma, ed., Martinus Nijhoff/Dr. W. Junk Publishers, The Hague (1984).
47. A. Stemler, Carbonic Anhydrase: Molecular Insights Applied to Photosystem II Research in Thylakoid Membranes, in: "Inorganic Carbon Transport in Aquatic Photosynthetic Organisms," J. Berry and W. Lucas, eds., American Society of Plant Physiologists, Rockwell, MD. In the Press (1984).
48. R. Barr, and R. Melhem, A. L. Lezotte and F. L. Crane, Stimulation of Electron Transport from Photosystem II to Photosystem I in Spinach Chloroplasts, J. Bioenerg. Biomemb. 12:197 (1980).
49. W. F. J. Vermaas and Govindjee, Bicarbonate Effects on Chlorophyll a Fluorescence Transients in the Presence and the Absence of Diuron, Biochim. Biophys. Acta 680:202 (1982).