

# Bicarbonate-Reversible Inhibition of Plastoquinone Reductase in Photosystem II

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In this paper the current status of the so-called *bicarbonate effect* is presented. Several chemicals (such as formate, azide, nitrite and nitric oxide) are known to inhibit the two-electron gate of photosystem II (PS II). A remarkable slowing down of  $Q_A^-$  reoxidation and an increase in equilibrium  $[Q_A^-]$  have been observed after the second or the subsequent, but not the first, flash when thylakoid membranes are treated with formate, etc. And, significantly, these effects are totally and uniquely reversed upon bicarbonate addition. The current hypothesis is that bicarbonate functions as a proton shuttle that stabilizes the binding niche of  $Q_B^-$  and stimulates plastoquinol formation. This bicarbonate effect must involve both the D1 and D2 proteins since various herbicide-resistant D1 mutants (*e.g.*, D1-S264A, D1-L275F), as well as some D2 mutants (*e.g.*, D2-R251S, D2-R233Q) have been found to be differentially sensitive to formate. The D2-arginine (233, 251) effects are specific since D2-R139H mutant and another mutant in which an extra arginine was inserted, between F223 and E224, behaves like the wild type. Data in the literature suggest that the bicarbonate binding must also involve Fe in the PS II  $Q_A-Fe-Q_B$  complex. In contrast, the  $Q_A-Fe-Q_B$  complex and the two-electron gate of both green and purple photosynthetic bacteria, including the M-E234G, Q and V mutants, are insensitive to bicarbonate-reversible inhibitors. We will also address the question of the nature of the active species involved and the possible role of bicarbonate *in vivo*.

## Introduction

Photosynthetic organisms are divided into two groups: oxygenic (higher plants, all algae and cyanobacteria) and anoxygenic (green and purple photosynthetic bacteria). The common feature of all these organisms is that they first convert light energy into oxidation-reduction (chemical) energy at reaction center (bacterio) chlorophyll molecules which have been named as P680, P700, P865, P840, etc. (the numbers representing their long-wavelength absorption peaks in nanometers). Photosystem II (PS II) reaction center (RC) Chl *a* P680 is uniquely different from those of PS I (Chl *a* P700), of purple and green non sulfur photosynthetic bacteria (BChl *a* P865, P870) and of green sulfur bacteria (BChl P840) since its redox potential of +1.1 V [1, 2] is the only one capable of oxidizing water to molecular  $O_2$  (average  $E_{m,7} \approx +0.9$  V). Another major difference between PS II and other reaction centers is that it contains Mn

(for the first discussion on Mn binding in PS II RC, see [3]). In spite of the well-known similarity in the proteins (D1/D2 with L/M [4, 5]) and in the functioning of the two-electron gate [6] on the electron acceptor side, PS II is known to be different from the RC of photosynthetic bacteria in other aspects as well: (1) accessibility and the reactivity of the non-heme iron: The  $Fe^{2+}$  can be oxidized in PS II by ferricyanide and the  $Fe^{3+}$ , thus formed, can be reduced by illumination of PS II [7, 8]; also, only the PS II non-heme Fe can bind to NO [9], the  $E_{m,7}$  of PS II Fe being +400 mV instead of  $> +550$  mV in bacterial RC; (2) replacement of  $Q_A$  with other quinones is more difficult in PS II than in bacterial RC; (3) one of the Chl *a* molecules of P680 is oriented  $30^\circ$  to the membrane and P680 appears to be more of a monomer than the bacterial RC electron donor which is clearly a dimer (see [10]); and (4) there are remarkable differences in the sensitivity to various herbicides (see [11] and [12]). In this paper, we will deal with still another difference: a *bicarbonate effect* that exists only in PS II RC from plants [13, 14] and cyanobacteria [15, 16], but not in RC from green [17] or purple [18, 19] photosynthetic bacteria. This effect can be easily observed as a bicarbonate-reversible inhibition of plastoquinone reductase in PS II.

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### The Bicarbonate Effect

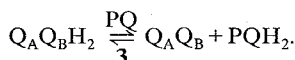
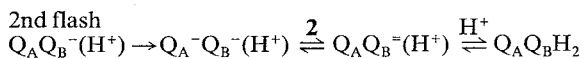
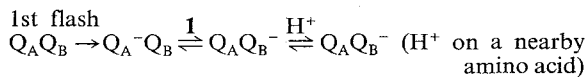
Warburg and Krippahl [20] showed that the Hill reaction was inhibited by CO<sub>2</sub> removal and strongly stimulated by the addition of CO<sub>2</sub> at low partial pressure under conditions where no reduction of CO<sub>2</sub> occurred. This phenomenon is what we call the *bicarbonate effect*. Although Warburg [21] cited this phenomenon as evidence for his unusual scheme in which O<sub>2</sub> evolved from CO<sub>2</sub>, observations in our laboratory have clearly established that a major effect of bicarbonate is on the electron acceptor side of PS II (see reviews [13, 14]). This effect is best observed by using formate as a bicarbonate-reversible inhibitor; formate indeed removes and releases bound bicarbonate [22].

Several other inhibitors besides formate have been used to study the bicarbonate-reversible inhibition of plastoquinone reductase in PS II. These include azide, nitrite [23, 24] and nitric oxide [25] which also inhibit the reoxidation of Q<sub>A</sub><sup>-</sup>, as monitored by Chl *a* fluorescence yield decay. Diner and co-workers [9, 11, 25], through their work on nitric oxide, have established the role of non-heme Fe in the *bicarbonate effect*. The effects of other inhibitors, however, may suggest the role of amino acids in the Q<sub>A</sub>-Fe-Q<sub>B</sub> binding niche.

Any complete hypothesis of the *bicarbonate effect* will have to involve an understanding not only of the effects on the electron acceptor side, but also on the electron donor side that appears to lie between the electron donor "Z" and the electron acceptor Q<sub>A</sub> [26]. Until new data on the Z to Q<sub>A</sub> step is available, we will concentrate on the steps on the electron acceptor side.

### Two-Electron Gate and the Protonation Hypothesis

Plastoquinone reduction in PS II occurs in two steps (see *e.g.*, Wraight [6]). Ignoring the donor side, this can be written as:



If the bicarbonate-reversible inhibitors were to inhibit the reaction labeled as **1**, we expect to find

full effect after the illumination of the sample by flash 1. Almost all of the available data show the full inhibitory effect only after the second and subsequent, not first, flash [27, 28]. This has been taken to suggest that the protonation step before the 2nd flash is inhibited by the inhibitor. Since bicarbonate fully reverses the effect, bicarbonate has been assigned the role of a proton shuttle [14].

The concept that bicarbonate plays a role in protonation has been supported also by proton measurements [29–31]. However, we still need to understand (1) our early results [32], although with different dark time between flashes, in which the major inhibition was seen after the 3rd, not the 2nd flash, suggesting a blockage in the binding and the unbinding of PQ and PQH<sub>2</sub>, respectively and (2) experiments in which even the first flash effects are rather large [33] although still smaller than the second flash effects. This last point is further heightened by our recent observations on chloroacetates where first flash effects are observed at pH 7.5, some of which are reversed by bicarbonate [26]. Thus the current protonation hypothesis needs to be refined further to accommodate these observations. Perhaps, we must consider the role of extra negative charge on bicarbonate to be near Q<sub>A</sub> or Q<sub>B</sub> affecting these reactions.

### The Binding Niche

The interaction of herbicide and bicarbonate binding has been known from the time the binding of herbicide was shown to be affected by bicarbonate and *vice versa* [34–36]. Since herbicides are known to bind to the D1 protein, it was obvious that the bicarbonate binding niche must involve the D1 protein. Khanna *et al.* [34] showed that a D1 mutant (S264G) had an altered binding of bicarbonate or the bicarbonate-reversible inhibitor formate. Recent experiments with various herbicide-resistant D1 mutants in *Chlamydomonas reinhardtii* [37, 38], *Synechocystis sp.* PCC 6714 [39] and *Synechococcus sp.* PCC 7942 [40], altered in single or double amino acids in helix IV or V or in the interhelical loop, have shown that there is a hierarchy in the bicarbonate-reversible inhibition (Fig. 1, top and middle panels): the mutants S264A/F255L and S264A were more sensitive, and L275F and A251V/F211S were more resistant to formate than the wild type. On the other

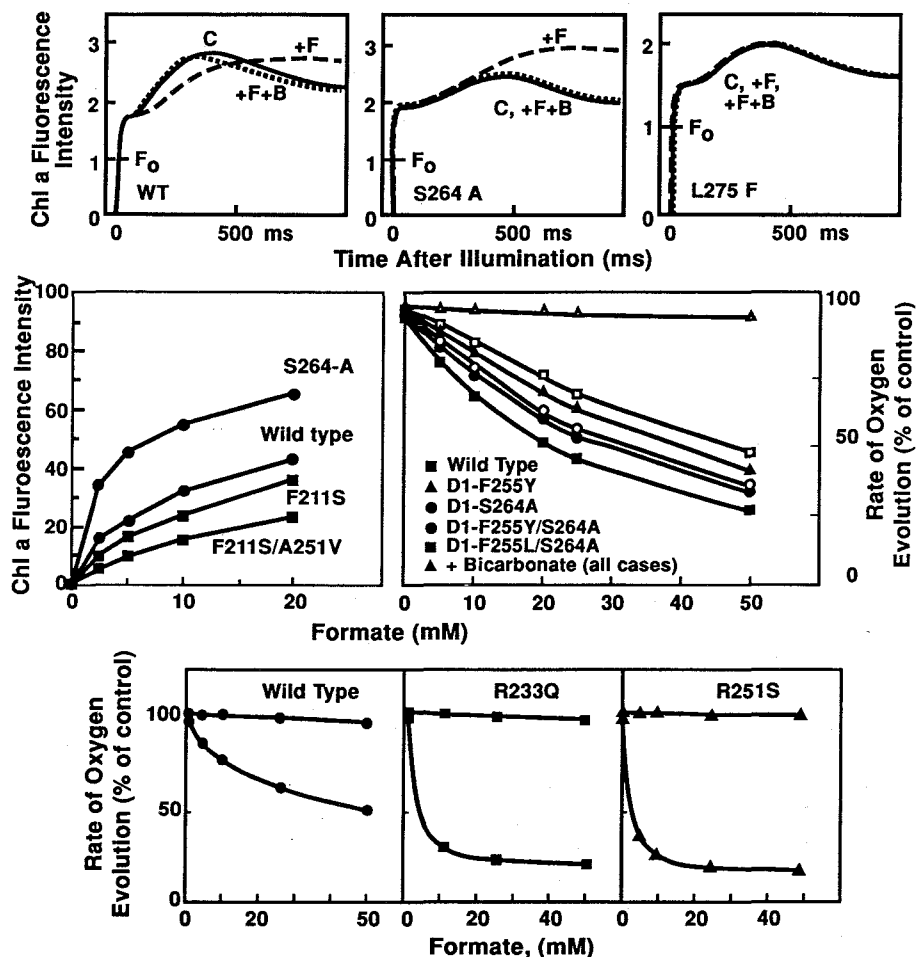


Fig. 1. (Top panel): Chl *a* fluorescence transients of wild type, D1-S264 A or D1-L275F *Chlamydomonas reinhardtii* cells (C) treated with 100 mM formate (F) and F + 10 mM bicarbonate (B). The  $F_0$  is the initial fluorescence intensity measured with a camera-shutter instrument; the true  $F_0$  is much lower than the measured  $F_0$  (after [37]). (Middle panel): left: Chl *a* fluorescence yield as a function of [formate] in wild type and the D1 mutant (S264 A, F211 S or F211 S/A251 V) cells of *Synechocystis sp.* PCC 6714 (after [39]); right: rate of oxygen evolution as a function of [formate] in wild type and the D1 mutant (F255 Y, S264 A, F255 Y/S264 A or F255 L/S264 A) cells of *Synechococcus sp.* PCC 7942 (after [40]). (Bottom panel): Rate of oxygen evolution as a function of [formate] in wild type or the D2 mutant (R233 Q or R251 S) cells of *Synechocystis sp.* PCC 6803 (after [42]).

hand, V219I and F255Y were more like the wild type. This differential sensitivity holds the clue to the role of bicarbonate binding and its function. We suggest that S264 must be intimately involved in the protonation mechanism of the two-electron gate and L275 may play a role in the conformation of the protein. We further suggest (also see [14]) that D1-R269 may play a crucial role in bicarbonate/formate binding. Thus, J. Xiong, in my laboratory, is in the process of constructing site-directed mutants at this site.

D1, that harbors  $Q_B$ , is intimately bound to D2, that harbors  $Q_A$ , whereas the non-heme iron is suggested to be connected to 4 histidines, 2 each in D1 and D2 (see Trebst [41]). As mentioned earlier, bicarbonate binding to iron seems pretty certain. However, the involvement of the D2 protein in the bicarbonate effect became obvious when Cao *et al.* [42] showed that D2-R251 S and D2-R233 Q were ten times more sensitive to the inhibitory formate than the wild type *Synechocystis sp.* PCC 6803 cells (Fig. 1, bottom panel). This effect is not due

Table I. The steady-state oxygen evolution rate and  $I_{50}$  (with formate) of wild type and two D2 mutants of *Synechocystis* 6803<sup>+</sup> (after Govindjee, S. Carpenter, J. Cao, and W. F. J. Vermaas, unpublished data, 1990).

Cyanobacteria	O <sub>2</sub> evolution rate [ $\mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$ ]	$I_{50}$ [mM]	Recovery with 10 mM HCO <sub>3</sub> <sup>-</sup> (%)
Wild type	172	49	92
D2-R 139H*	170	48	94
211**	165	50	94

\* This mutant was constructed by the author (Govindjee) in W. F. J. Vermaas' laboratory, with the help of Shelly Carpenter (for Methods, see [42]).

\*\* Mutant provided by W. F. J. Vermaas: here, an extra arginine had been inserted between F 223 and E 224 in the D2 protein.

† A combination of 0.5 mM 2,5-dimethyl-*p*-benzoquinone and 1 mM K<sub>3</sub>Fe(CN)<sub>6</sub> was used as electron acceptor; 20  $\mu\text{M}$  2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone was used to block electron transfer between the plastoquinone pool and PS I. Reaction mixture contained: 0.1 M sucrose, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1  $\mu\text{M}$  gramicidin D and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (pH 6.8).

to some non-specific effect of arginines since the D2-R 139H, which I had constructed in W. F. J. Vermaas' laboratory, behaves like the wild type *Synechocystis* sp. PCC 6803 (Table I). Furthermore, a mutant No. 211, which had an extra arginine inserted between D2-F 223 and D2-E 224, also behaves like the wild type (Table I). It can, thus, be suggested that D2-R 251 and D2-R 233 may be involved in the binding niche for bicarbonate; they may somehow stabilize bicarbonate binding to PS II. On the other hand, Diner and co-workers (see [11]) have obtained several D2-K 264X and

D2-R 265X mutants that are drastically slowed in their reoxidation of Q<sub>A</sub><sup>-</sup> even in the absence of bicarbonate-reversible inhibitors. Thus, these amino acids may serve to bind bicarbonate, only H-bonding being considered. Fig. 2 is a possible model of some of the amino acids in the binding niche of bicarbonate/inhibitors.

The question about the exact binding niche of bicarbonate still eludes us. Thus, another approach, besides the use of site-directed mutants, has been attempted. Xu [26] and Xu *et al.* [43] have used halogenated acetates. Table II shows that the

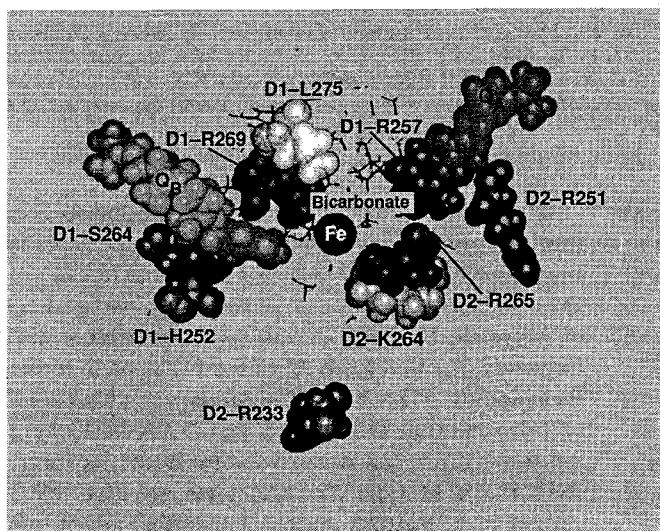


Fig. 2. A speculative model of the binding niche of bicarbonate in a portion of the D1 and D2 polypeptides of PS II that includes the Q<sub>A</sub>FeQ<sub>B</sub> region. The circle in the center is the non-heme iron. The reader can get some feeling for distances as Fe is assumed to be about 7 Å from Q<sub>A</sub> and Q<sub>B</sub>. D1-S264 and D1-L275 are known to be involved in herbicide resistance; these as well as D2-R233, D2-R251, D2-K264 and D2-R265 have been shown to be involved in the bicarbonate effect; furthermore it has been suggested that D1-R257 and D1-R269 are also involved in the bicarbonate effect. Bicarbonate is suggested to be liganded to Fe and H-bonded to several amino acids similar to that in lactoferrin (see Fig. 3). This figure was constructed by C. Gibas and Govindjee (unpublished, from a PS II model of H. H. Robinson, C. Yerkes, and A. R. Crofts, also unpublished).

Table II. Correlation of the inhibitory effect of halogenated acetates on  $[Q_A^-]$  decay in spinach thylakoids with their physical properties\*.

Chemicals	Calculated lifetime $Q_A^- \rightarrow Q_B^{(-)}$ ( $\mu$ s)	Calculated $[Q_A^-]$ decay		Bicarbonate reversibility	$pK_d$	Properties of chemicals	
		Nos. related to equilibrium of $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ (slow/fast components)				Dipole moment (Debye)	Hydrophobicity index (log of partition coefficient)
Acetate	610	0.9	+++++	4.7	1.6	-0.33	
Monofluoroacetate	650	0.8	++++	2.6	3.1	-0.27	
Monochloroacetate	845	1.8	++++	2.8	3.3	+0.32	
Monobromoacetate	860	1.4	+++	2.9	3.1	+0.64	
Dichloroacetate	920	3.2	++	1.3	2.5	+1.33	
Trichloroacetate	1700	24.0	+	0.7	2.1	+1.54	

Correlation of increase in lifetime of electron flow from  $Q_A^-$  to  $Q_B^{(-)}$  and equilibrium  $[Q_A^-]$  appears to be pretty good with the partition coefficient and, thus, with the hydrophobicity.

\* Based on data in ref. [26] and [43].

inhibition of  $Q_A^-$  to  $Q_B^-$  reaction and of equilibration of  $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$  is correlated well with the hydrophobicity (*i.e.*, the partition coefficient) of the halogenated acetates. Furthermore, the bicarbonate reversibility decreases as the hydrophobicity increases. The relationship of the effects of these halogenated acetates to hydrophobicity is reminiscent of a similar relationship of the inhibitory effects of herbicides and their hydrophobicity [44]. The nature of this clue also needs to be explored: whether it only relates their ability to partition into the membrane and/or to the physico-chemical nature of the binding site.

Table III. A comparison of the effects of some weak acid anions on PS II (inhibition) and on *R. sphaeroides* L-D213N mutant (stimulation) on  $Q_A^-$  to  $Q_B^-$  reaction.

Chemicals	PS II	<i>R. sphaeroides</i> L-D213N Mutant
	$C_{1/2}$ (at 2 ms)*	$C_{1/2}$ (total)**
Formate	10 mM	80 mM
Nitrite	10 mM	23 mM
Azide	20 mM	24 mM

\* Concentration of chemical required to get half maximal effect on second electron flow, 2 ms after the flash (estimated from Cao and Govindjee [24]).

\*\* Concentration of chemical required for two-fold acceleration of second electron flow (Takahashi and Wraight [46]).

We have been looking for a model for bicarbonate binding in PS II. The only other known Fe-(bi)carbonate protein is (human) lactoferrin [45]. Fig. 3 shows a diagram of this protein based on its available X-ray structure [45]. What is of interest to us is that carbonate has a bidentate ligand to Fe, is H-bonded to an arginine and three other amino acids. We suggest that similar arrangements may exist in PS II.

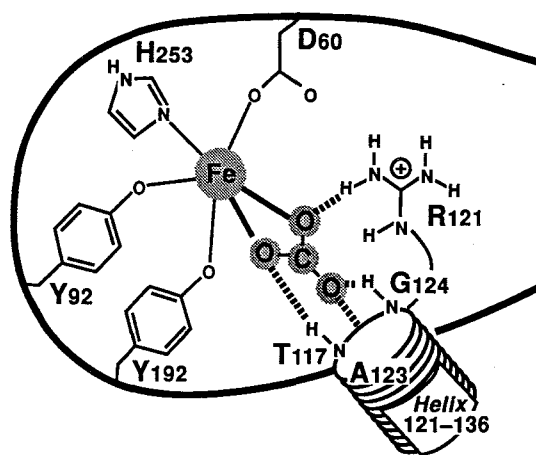


Fig. 3. Schematic representation of the iron and (bi)carbonate binding region in lactoferrin, the only other Fe-(bi)carbonate protein known to the author. (Bi)carbonate has a bidentate ligand to Fe and is H-bonded to arginine and several other amino acids (after Andersson *et al.* [45]).

### Comments on Photosynthetic Bacteria

As noted earlier, photosynthetic bacteria do not show any bicarbonate-reversible inhibitor effects. Neither nitric oxide [9] nor formate [18] inhibits the two-electron gate of photosynthetic bacteria. Thus, it is obvious that bicarbonate, as such, is not a strict requirement for the operation of the two-electron gate of quinol formation. This absence of the requirement does not mean that the bicarbonate effect is not important in the functioning of PS II.

Glutamate (M-E234 in *R. sphaeroides* or M-E232 in *R. viridis*) was suggested to replace bicarbonate in photosynthetic bacteria [4]. Wang *et al.* [19] tested this hypothesis. They showed that M-E234V, M234Q and M234G mutants of *R. sphaeroides* are not only unaffected by formate, but show no significant differences from those in the wild type in their  $Q_A^-$  to  $Q_B^{(-)}$  reactions, dispelling the belief that M-E234, liganded to Fe, has a crucial role to play in the bicarbonate effect as well as in the normal electron flow on the electron acceptor side.

Interestingly, Takahashi and Wraight [46] observed that in the L-D213N mutant of *R. sphaeroides*, which normally shows the characteristics of a bicarbonate-depleted PS II, formate azide and nitrite etc. accelerate electron flow, and accompanying protonation, during  $Q_A^-$  to  $Q_B^-$  reaction. Table III shows a comparison of the concentration dependence of this acceleration effect with the inhibitory effect on PS II. Both effects are in the mM range. It appears that these weak acid anions must not bind in photosynthetic bacteria, but simply act as protonophores. However, they must bind in PS II in such a way that their proton groups are tied and unavailable for protonation. In contrast, bicarbonate, that has an extra proton group even when it is bound, can provide  $H^+$ s for  $Q_B^-$  stabilization. Thus, the difference between PS II and photosynthetic bacteria may lie in the former having a binding site for bicarbonate, and the latter not.

### Concluding Remarks

#### *Nature of active species*

On the basis of the pH dependence of the bicarbonate effect under equilibrium conditions, Blu-

baugh and Govindjee [47] concluded that bicarbonate, not  $CO_2$ , is the active species. Unfortunately, in view of the absence of knowledge about the role of the PS II RC protein in affecting these results, it is not possible to exclude the role of  $CO_2$  in this phenomenon. In all likelihood,  $CO_2$  is the diffusing species since  $CO_2$  stimulates the Hill reaction faster than  $HCO_3^-$  [48]. Thus, we need to consider both  $CO_2$  and  $HCO_3^-$  while discussing the *bicarbonate effect*.

#### *Essential versus non-essential activator*

Whether bicarbonate is an essential requirement for PS II, *i.e.*, without bicarbonate, no electron flow is possible, or whether it is a non-essential activator is a very important question to answer. This question is complicated by the possibility that the non-essential nature may be observed only because an alternate proton pathway becomes operative which takes over the proton shuttle function when either enough  $H^+$ s are available or bound water becomes functional. However, when bicarbonate is bound, it not only serves to keep the inhibitory anions away from the binding site, but it provides  $H^+$ s as suggested earlier [14].

Jursinic and Stemler [49] showed that in thylakoids suspended in  $CO_2$ -depleted medium deprived of anions, the electron transport activity is almost normal. Furthermore, in such preparations injection of formate was shown not to lead to measurable  $CO_2$  release. They, thus, concluded that bicarbonate is not a *requirement* for Hill Reaction under these conditions. A skeptic may raise the question: Since the sample is free of most  $CO_2$ -bound sites, wouldn't the release of "tightly bound"  $CO_2$  at the reaction center lead to its re-binding at a large number of empty sites and slow the kinetics of  $CO_2$  release that can be measured in the time frame of the experiment? Both a quantitative calculation of the expected amount of  $CO_2$  released and the time course of this  $CO_2$  release are required before this "negative" result is used to reach a final conclusion. Additionally, a low anion condition may lead to a larger availability of  $H^+$ s and/or bound water. It is considered likely that bound water may provide protons instead of bicarbonate under these conditions. However, at the alkaline pH (stroma pH is 8), plenty of bicarbonate is bound and must serve as a proton shuttle, as suggested.

Cao *et al.* [40] have attempted to analyze the rate of the Hill reaction in several D1 mutants of *Synechococcus sp.* PCC 7942 by plotting the inverse of the rate of Hill reaction as a function of formate concentrations in samples to which different concentrations of bicarbonate were added (Dixon plots). The essential and the non-essential models predict different results for the rate of Hill reaction when the added [formate] is zero. However, this distinction between the two models can be made only in samples thoroughly depleted of CO<sub>2</sub> to which low concentrations of activator (bicarbonate) are added under conditions that resemble those *in vivo* (*i.e.*, alkaline pH). It is now a challenge to obtain rigorous experimental conditions to test the two models.

#### *Role in vivo*

Since the quantum yield of PS II photochemistry and the quantum yield of CO<sub>2</sub> assimilation are almost linearly related [50], the productivity of plants growing in shade, and of shaded leaves in a canopy, etc. must be affected by PS II reactions. The importance of bicarbonate, thus, becomes obvious since it modulates PS II reactions. Ireland *et al.* [51] showed that in maize leaves, lowered [CO<sub>2</sub>] leads to a reduction in the Q<sub>A</sub><sup>-</sup> to Q<sub>B</sub> reactions. Experiments of Mende and Wiessner [52] further showed the importance of CO<sub>2</sub> in PS II reactions in algal cells. Blubaugh and Govindjee [14] have pointed out that at the alkaline pH of the stroma (the condition *in vivo*) there should be plen-

ty of bicarbonate bound in ambient air as the K<sub>d</sub> is estimated to be about 40 μM.

Several possibilities exist for the function of CO<sub>2</sub>: (1) protection against destabilization of PS II (see *e.g.* [53]); (2) regulation of electron flow: when [CO<sub>2</sub>] level falls, CO<sub>2</sub> fixation is reduced and, thus, simultaneously decreased bound [CO<sub>2</sub>] in PS II would decrease O<sub>2</sub> evolution as well as the reducing power. The decreased [O<sub>2</sub>] would decrease photorespiration [11], and the decreased [reductant] would prevent the waste of components. Furthermore, there may be protection against photoinhibition, an idea being investigated in several laboratories.

It is my opinion that whether any of the above roles can be proven by further experiments, bicarbonate is a key component of *in vivo* PS II and serves to aid in protonation as well as electron flow reactions under *in vivo* conditions. Whether these effects are direct as we have imagined them to be, or whether they are indirect through stabilization reactions only, or both remains to be discovered. PS II particles in which bicarbonate depletion can be achieved without displacement by another anion [54] is a promising tool for answering the question of the essential *versus* non-essential activator model of CO<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>.

#### *Acknowledgements*

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