

# On the Requirement of Bound Bicarbonate for Photosystem II Activity

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In photosystem II of plants and cyanobacteria, but not in reaction centers of anoxygenic photosynthetic bacteria, formate is known to inhibit electron flow which is reversed fully upon bicarbonate addition. At issue has been an old controversy whether this effect is on the acceptor or the donor side of photosystem II (PS II). We present here data on chloroplast thylakoids for donor side effects, that is accompanied by acceptor side effects, from measurements on chlorophyll *a* fluorescence yield changes after light flashes 1–6. Further, sensitive differential infrared gas analyser measurements show that bicarbonate is indeed bound in both maize and pea thylakoid suspensions depleted of CO<sub>2</sub> without any inhibitor; here, high rates of electron flow are associated with the presence of a maximum of 0.8 to 1.25 (corrected for residual activity) CO<sub>2</sub> per photosystem II reaction center. It is suggested that bicarbonate bound to the acceptor side is required for photosystem II activity, both on the acceptor and the donor sides in the same experiment and in the same sample.

## Introduction

Carbon dioxide is required for photosynthesis as it is fixed by ribulose 1,5-bisphosphate carboxylase to produce carbohydrates. In addition, CO<sub>2</sub> (or HCO<sub>3</sub><sup>–</sup>) is also involved in photosynthetic electron transport. Although a major effect is on the acceptor side of photosystem II (PS II) (see e.g., Govindjee and van Rensen, 1993; Diner *et al.*, 1991; Blubaugh and Govindjee, 1988b), an effect on the donor side was advanced earlier by Stemler and coworkers (Stemler and Govindjee, 1974; Stemler,

1980). An effect on the donor side was recently confirmed (see e.g., El-Shintinawy and Govindjee, 1990; Klimov *et al.*, 1995). Incubation of plant thylakoids and cyanobacteria in media with high concentrations of anions (formate, acetate, azide, nitrate, etc.) or NO inhibits electron flow. This inhibition is reversed fully and uniquely by the addition of bicarbonate (see e.g., Good, 1963; Jursinic and Stemler, 1988; Diner *et al.*, 1991). Bicarbonate has been shown to be liganded to the non-heme iron in the Q<sub>A</sub>-Fe-Q<sub>B</sub> complex of PSII (Petrouleas *et al.*, 1992; Hienerwadel and Berthomieu, 1995). Hutchison *et al.* (1996) have shown that a mutation (D1-R-269G) on D1-arginine 269 on the acceptor side of photosystem II, suggested earlier to be a potential bicarbonate-binding site, led to drastic changes not only on the acceptor, but also the donor side of PS II. Thus, we have reinvestigated the effect of formate on the donor side of spinach thylakoids by measuring chlorophyll (Chl) *a* fluorescence yield after a light flash. We confirm the existence of a donor side effect as fluorescence yield at < 1 ms after flashes 1 and 2 (at pH 6) first decreases before increasing to a higher value.

Jursinic and Stemler (1992) discussed two hypotheses for this bicarbonate effect: (1) the “bound-bicarbonate” hypothesis: here, a high-affinity binding of bicarbonate is required for high

**Abbreviations:** Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; Fo, minimal chlorophyll *a* fluorescence yield when all Q<sub>A</sub> is in the oxidized state; F<sub>m</sub>, maximal chlorophyll *a* fluorescence yield when all Q<sub>A</sub> is in the reduced state; F<sub>v</sub>, variable chlorophyll *a* fluorescence yield that equals F<sub>t</sub> minus F<sub>o</sub>, where F<sub>t</sub> is chlorophyll *a* fluorescence at time *t* after a light flash; FeCy, potassium ferricyanide; IRGA, infra-red gas analyzer; P680, reaction center chlorophyll *a* of PSII; PSII, Photosystem II; Q<sub>A</sub>, primary plastoquinone electron acceptor of Photosystem II; Q<sub>B</sub>, secondary plastoquinone electron acceptor of Photosystem II; Z, electron donor to oxidized P680, P680<sup>+</sup>.

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rates of electron flow; and (2) the “inhibitory-anion” hypothesis, also called the “empty-site” hypothesis. Here, for high rates of electron flow, the binding site may be entirely free of any inhibitory anion, and bicarbonate may function only to displace the inhibitory anions. These two explanations differ in the residency state of the binding site for bicarbonate when normal (rapid) electron flow occurs. Jursinic and Stemler (1992) failed to demonstrate the presence of any  $\text{CO}_2$  in  $\text{CO}_2$ -depleted thylakoid suspensions in low (10 mM) NaCl medium that had high rates of electron flow. Thus, they concluded that the inhibitory-anion or “empty-site” hypothesis is correct, implying that bicarbonate is not a requirement for PSII reactions at this site. On the other hand, Cao *et al.* (1992), using a kinetic model, were unable to distinguish between what they had called the essential and non-essential activator models, respectively, equivalent to the “bound bicarbonate” and “empty-site” hypothesis. Here, we demonstrate that 0.8 to 1.25  $\text{CO}_2$  molecule is bound per reaction center of PSII in maize and pea thylakoids under sample preparation conditions similar, but may not be identical, to those used by Jursinic and Stemler (1992). A preliminary report on this observation was first made in a conference proceedings (deVos *et al.*, 1995). Our results lead to the qualitative conclusion that the “bound bicarbonate”, not the “empty-site”, hypothesis is correct. However, it is speculated that although in normal systems, bicarbonate is bound and is used to regulate protonation of PS II reactions (see e.g., Eaton-Rye and Govindjee, 1988; Blubaugh and Govindjee, 1988a), it could be replaced by water molecules. For a detailed model, see Xiong *et al.* (1996).

## Materials and Methods

### *Chlorophyll a fluorescence measurements*

#### Sample preparation

For chlorophyll (Chl) *a* fluorescence measurements, thylakoids were prepared as described below at 0–4 °C. Young fresh market spinach (*Spinacia oleracea*) leaves were ground in a medium containing 400 mM sorbitol, 50 mM NaCl, 1 mM EDTA (ethylenediamine tetraacetic acid) and 50 mM Hepes (N-3-Hydroxyethylpiperazine-N'-2-

ethanesulfonic acid) for 5 s in a Waring blender. The suspension was filtered through 12 layers of cheesecloth, and then spun at 5,000x *g* for 60 s to remove debris. The filtrate was then recentrifuged at 5,000 x *g* for 10 min, and the pellet of the last step was resuspended and osmotically shocked in a medium containing 50 mM NaCl, 5 mM  $\text{MgCl}_2$  and 10 mM Hepes (pH 7.8). The suspension, at a Chl concentration of 1 mM, was rapidly frozen to 77 K, in 0.5 ml plastic vials; they were used immediately after thawing.

#### Fluorescence measurements

To monitor the effects of formate on the acceptor and donor sides of PS II, Chl *a* fluorescence changes, in thylakoid membranes containing 10  $\mu\text{M}$  [Chl], were measured by an instrument described by Eaton-Rye and Govindjee (1988). The suspension medium was 400 mM sorbitol, 50 mM NaCl and 2 mM  $\text{MgCl}_2$ , and the pH of the medium was adjusted either with 20 mM Hepes (pH 7 to 7.5) or with 20 mM Mes (2-[N-morpholino]ethanesulfonic acid; pH 5.5 to 6.5). In the fluorescence experiments, single-turnover blue (CS 4–96) saturating flashes (EG & G FX-124 flash lamp, 2.5  $\mu\text{s}$  duration) were used as actinic (or, pump) light, whereas weak measuring blue flashes were used as monitoring (or probe) light. The initial “0” (or  $F_0$ ) level of Chl *a* fluorescence yield was measured with a weak flash in 5 min dark-adapted thylakoids; changes in the variable fluorescence yield were measured after saturating actinic flashes, at 685 nm (bandwidth, 10 nm; photomultiplier, S-20 response, EMI 9558, protected by red CS 2–61 filter). Concentration of  $Q_A$ , the first plastoquinone electron acceptor of PS II, was calculated as described by A. Joliot and P. Joliot (1964). Time dependence of the formate effect, that allowed us to separate the donor and acceptor side effects, was done by varying the time of mixing of the thylakoids with 100 mM formate, as described elsewhere (Xu *et al.*, 1991).

#### *Differential infrared gas analysis*

#### Sample preparation

For these measurements, a different protocol was followed in order to compare our results with those obtained by Jursinic and Stemler (1992).

Broken chloroplasts (thylakoids) were prepared from 10 to 15 day old pea plants (*Pisum sativum* L. var. Rondo) and 7 to 10 day old maize plants (*Zea mays* L.) grown in climate-controlled rooms. Peas were grown at a light intensity of  $220 \mu\text{E m}^{-2} \text{s}^{-1}$  and at  $20^\circ\text{C}$ , whereas maize was grown at a light intensity of  $130 \mu\text{E m}^{-2} \text{s}^{-1}$  and at  $25^\circ\text{C}$ . Thylakoids were isolated as described by Snel and van Rensen (1984), suspended in a medium containing 400 mM sorbitol, 20 mM tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM  $\text{MgCl}_2$ , 2 mM Na-ascorbate and 0.2% (w/v) bovine serum albumin, and stored at  $-80^\circ\text{C}$ . Thylakoids were thawed on ice before use.

### Removal of bicarbonate

In order to remove dissolved  $\text{CO}_2/\text{HCO}_3^-$ , the reaction medium was boiled and bubbled with nitrogen gas as the temperature was decreased back to about  $25^\circ\text{C}$ . The  $\text{CO}_2$  concentration of the boiled and bubbled medium was measured indirectly by  $\text{O}_2$  concentration measurement. The  $\text{O}_2$  concentration of the medium used for the experiments was between 7 and  $15 \mu\text{M}$ . To avoid  $\text{CO}_2$  contamination when handling samples, all transfers were made with gas tight syringes into covered centrifuge tubes, vials, flasks or reaction vessels that had been pre-flushed with nitrogen.

### Pretreatment and other checks

Maximum rates of  $\text{O}_2$  evolution in continuous saturating light were measured as described by Snel and van Rensen (1984). Thylakoids were pretreated exactly as described by Jursinic and Stemler (1992), centrifuged at  $1000 \times g$  for 8 min, and incubated in the dark at  $20^\circ\text{C}$  for 30 min in a reaction medium that contained 50 mM sodium phosphate and 10 mM NaCl at pH 6.4 at a concentration of  $50 \mu\text{g Chl.ml}^{-1}$ . Then, after collection by centrifugation and resuspension in fresh  $\text{CO}_2$ -depleted reaction medium, 10 mM  $\text{NaHCO}_3$  was added to one sample and the thylakoids incubated for 2 min in the dark. After the addition of 5 mM  $\text{NH}_4\text{Cl}$ ,  $40 \mu\text{M}$  DCBQ (2,6 dichlorobenzoquinone) and 0.4 mM FeCy (ferricyanide),  $\text{O}_2$  evolution was measured. To a second sample no additions were made, and to a third sample 100 mM formate was added and handled in the same way.

### Infraredsystem

As was already indicated by Jursinic and Stemler (1992), it is difficult to measure small changes in  $\text{CO}_2$ -concentration in the gas phase above a thylakoid suspension, using a closed LI-COR infrared gas analyzer system. These authors took special precautions (such as covering the entire instrument with plastic) to avoid, e.g., leakage. To circumvent this problem, we, however, chose to use an open infrared gas analyzing system (Fig. 1), and to inject samples taken from the gas

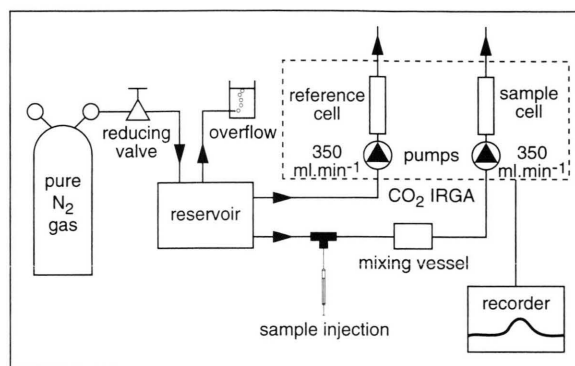


Fig. 1. A block diagram of the Infra Red Gas Analyzer (IRGA) to measure  $\text{CO}_2$  concentrations of samples taken from the gas phase above the thylakoid suspension, or above a reaction medium, and injected into the system.

phase above the thylakoid suspension. Carbon dioxide concentrations in the gas phase above the samples were measured with a type 225-MK2 infrared gas analyzing system (IRGA; Analytical Development Co. Ltd., Hertsfordshire, England). This IRGA system (Fig. 1) is based on a differential measurement method. In both the sample and the reference cell the reference gas (pure  $\text{N}_2$ ) is passed. The difference in the concentration of  $\text{CO}_2$  between the sample and the reference cell causes a difference in absorption of IR radiation. This exerts a difference in pressure between the measuring units (which are separated by a membrane) of both cells. This pressure difference is measured. Calibration of the IRGA was carried out with a calibration gas which contained 400 ppm  $\text{CO}_2$  in  $\text{N}_2$  gas. **Figure 2** shows perfect linearity in the response of our system. The flow rate of reference  $\text{N}_2$  gas through both the analyzing and the reference cells was  $350 \text{ ml min}^{-1}$ . Because of the positive pressure in our system, we

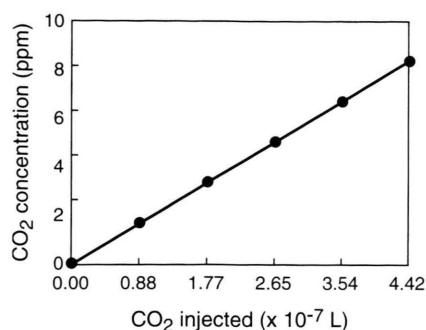


Fig. 2. Calibration curve relating the volume of injected CO<sub>2</sub> with ppm CO<sub>2</sub> measured by the IRGA.

encountered no problem of leakage of CO<sub>2</sub> into the analyzer.

Thylakoids were incubated at 20 °C in the dark for 30 min in a reaction medium containing 50 mM sodium phosphate and 10 mM NaCl at pH 6.4 at a concentration of 50 µg Chl.ml<sup>-1</sup>, whereafter they were collected by centrifugation at 1000 x g for 8 min and resuspended in 3.8 ml reaction medium at a concentration of 4.2 mg Chl.ml<sup>-1</sup>. The thylakoid suspension was transferred into a septum vial with a magnetic stir bar. To facilitate equilibration between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, 100 µl of carbonic anhydrase solution (20 µg · ml<sup>-1</sup>, pH 6.4) was added to a final concentration of 0.5 µg.ml<sup>-1</sup>. To examine whether the high affinity binding sites of the pre-treated thylakoids were depleted of bicarbonate or not, 100 µl of CO<sub>2</sub>-depleted 4 M sodium formate solution at pH 6.4 was injected to a final concentration of 100 mM. Both the carbonic anhydrase and the formate solution were prepared in CO<sub>2</sub>-depleted reaction medium at pH 6.4 and stored in septum vials. The carbonic anhydrase solution was kept on ice. The gas volume above the thylakoid suspension in the septum vial was 4.8 ml. The thylakoid suspension was incubated at 20 °C for 15 min in room light and was continuously stirred during the incubation period. A sample of 0.3 ml was taken from the gas phase above the thylakoid suspension and its CO<sub>2</sub> content was analyzed by injecting the sample into the IRGA via a rubber septum into the gas stream which then entered the analyzing sample cell (Fig. 1). We also measured controls in which, instead of thylakoid suspension, reaction medium was incubated. Further controls with boiled thylakoids were also tested for the CO<sub>2</sub> release.

## Results and Discussion

### *The donor side effects*

Warburg and Krippahl (1958, 1960) are to be credited with the discovery of the requirement of CO<sub>2</sub> in the Hill reaction by chloroplasts (see Blubaugh and Govindjee, 1988b for a historical description); O. Warburg (see Warburg, 1964) insisted that this phenomenon supported the idea that CO<sub>2</sub>, not H<sub>2</sub>O, was the source of O<sub>2</sub>. Govindjee and coworkers (Wydrzynski and Govindjee, 1975; Govindjee *et al.*, 1976; Jursinic *et al.*, 1976; Khanna *et al.*, 1981; see a review by Vermaas and Govindjee, 1982) were the first to establish that there was a definite bicarbonate effect on the electron acceptor side of photosystem II. Formate inhibited the electron flow on the acceptor side, and bicarbonate relieved this inhibition. However, theories and experiments continued to be presented by Stemler and coworkers for a donor side bicarbonate effect (see Stemler and Govindjee, 1974; and a review by Stemler, 1982); these were not generally accepted mainly because of the large effects on the acceptor side of PS II, obtained at high concentrations of formate. Mende and Wiesner (1985) suggested that CO<sub>2</sub>-depletion affects both the oxygen evolving and the electron acceptor side in intact algal cells. El-Shintinawy and Govindjee (1990) established that in contrast to long-term formate treatment, short-term formate treatment of spinach leaf discs led to a decrease in Chl *a* fluorescence yield instead of an increase in Chl *a* fluorescence yield as expected from an effect on the acceptor side of PS II. El-Shintinawy *et al.* (1990) reported a similar effect in *Chlamydomonas reinhardtii* cells. These authors were, however, unable to establish the exact location of the site of this effect; they suggested that the effect they observed was between the hydroxylamine donation site and Q<sub>A</sub>. It were Klimov *et al.* (1995) (also see Wincencjusz *et al.*, 1996) who convincingly showed that in PS II membrane fragments, a bicarbonate effect exists on the donor side of PS II in addition to the well known effect on the acceptor side. This effect appears easily or is the first one to appear, i.e., at lower concentration of formate, and appears sooner at the higher concentrations of formate, just as in the experiment of El-Shintinawy and Govindjee (1990) in leaf discs. Below, we show (i) that at low pH (e.g., pH 6), maximal



fluorescence ( $F_m$ ) in spinach thylakoid membranes is lower with formate, than without formate, suggesting an inhibition on the donor side of photosystem II; and (ii) depending upon the time of mixing of formate with the sample, this effect appears first followed by the usual acceptor side effect.

**Figure 3** shows the effect of formate addition on the Chl *a* fluorescence changes in spinach thylakoids, at pH 6, up to 2 ms after flash 1, 2, 3, 4, 5 or 6. Decrease in variable fluorescence, at 80  $\mu$ s after the flash, is observed after all the flashes, but it is a larger effect after 4th–6th flashes than after earlier flashes. Although its significance is not obvious, this may imply a multiphoton requirement for the full effect. A lowered  $F_m$  is easily explained by a limitation on the donor side and thus in either an increase in the quencher [P680<sup>+</sup>] and or in [ $Q_A$ ] (Shinkarev and Govindjee, 1993). This lowering is

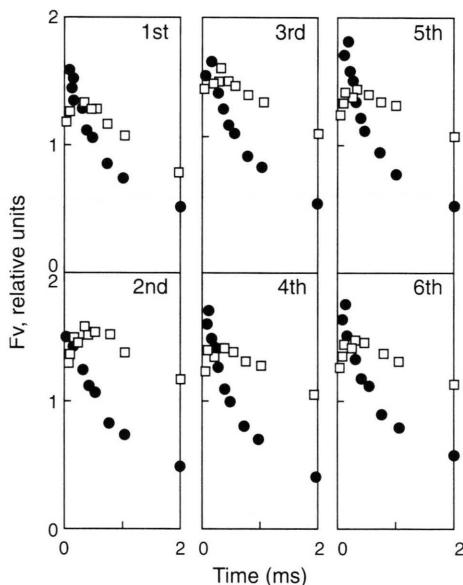


Fig. 3. Chlorophyll *a* fluorescence changes up to 2 ms after a series of single-turnover saturating flashes (1, 2, 3, 4, 5 and 6). Spinach thylakoids at pH 6. Closed circles, untreated; open squares, 100 mM formate.  $F_v$  refers to variable fluorescence. Time for dark adaptation and formate treatment was 15 min. Reaction medium contained 400 mM sorbitol, 50 mM NaCl, 2 mM  $MgCl_2$  and Mes. The addition of 20 mM bicarbonate totally reversed the formate effect to the control value. The first data points approach the  $F_m$  value; note that formate decreases this value due to inhibition of electron flow from the donor side to P680<sup>+</sup>, followed by a slowing down of the fluorescence decay due to inhibition of electron flow on the acceptor side of PSII.

followed by a slowing down of the fluorescence decay, the usual electron acceptor side effect.

**Figure 4** shows the time course of formate binding in spinach thylakoids as monitored by Chl *a* fluorescence yield at 1 ms after the 1st or the 2nd flash as a function of the mixing time after the injection of a formate solution, at several pHs. The change in  $Q_A^-$ , [ $Q_A^-$ ] (formate) minus [ $Q_A^-$ ] (control), reflects the fraction of PSII in which formate is bound. The increases in the above parameter beyond the base line are due to acceptor side effects. However, the decreases below the baseline, that appear at the early mixing time, are due to an effect of formate on the donor side. This effect was clearly observed at pH 6. It confirms the dual effect of formate on both the acceptor and donor side of PSII through independent experiments on spinach thylakoids (see Mende and Wiessner, 1985; El-Shintinawy and Govindjee, 1990; El-Shintinawy *et al.*, 1990). At 100 mM formate, donor side effects are overshadowed by the acceptor side effects at all other pHs. At lower concentrations of

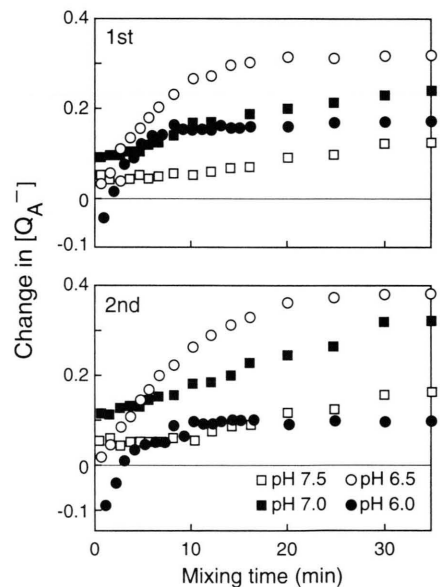


Fig. 4. Time dependence of formate binding at different pHs, as measured by Chl *a* fluorescence yield 1 ms after flashes 1 and 2. [ $Q_A^-$ ] was calculated without any consideration for any quencher other than  $Q_A$ . Different symbols indicate different pH (see key in the panel). Other experimental details are given in the legend of Fig. 3. Note that even with 100 mM formate, fluorescence decrease can be observed at pH 6 at early mixing time. The maximum effect on the acceptor side occurs at pH 6.5 as already known.

formate donor side effects are observed at other pHs also (see e.g., fluorescence transient measurements in intact cells and in chloroplasts, El-Shintinawy *et al.*, 1990; Stemler and Govindjee, 1974).

*Infrared gas analysis: CO<sub>2</sub> release*

Stemler (1989) and Jursinic and Stemler (1992) concluded that PSII electron flow can proceed normally when the bicarbonate binding site at PSII is empty of all monovalent anions, including bicarbonate. Govindjee *et al.* (1991b) had challenged Stemler's conclusion at pH 6 (1989), based on the fact that there was much more CO<sub>2</sub> release at pH 6.5 than at pH 6. The conclusion of Jursinic and Stemler (1992) was, however, based on the following experiment, done at pH 6.4.

In maize thylakoids, incubated in the dark for 30 min in a CO<sub>2</sub>-free medium (pH 6.5) containing 10 mM NaCl, the rate of O<sub>2</sub> evolution without the addition of bicarbonate was found to be only 5% lower than when bicarbonate was added two min before the start of the measurement. This must have been an almost uninhibited condition, in which the bicarbonate binding site has bicarbonate bound according to the bound-bicarbonate hypothesis, and is empty, according to the inhibitory-anion hypothesis. A choice in favour of the empty-site hypothesis was made when Jursinic and Stemler (1992) did not observe *any* CO<sub>2</sub> release after injection of 100 mM formate to thylakoids. Because there occurred an unidentified, what they called, respiratory process in their spinach and pea thylakoids, they did not use these samples.

In this work we have repeated this critical experiment. First, we could not find significant differences between CO<sub>2</sub> and O<sub>2</sub> exchanges in peas and maize in darkness as reported by Jursinic and Stemler (1992). We observed a small inhibition (10 -13%) after incubation of pea thylakoids in the dark for 30 min in a medium containing 10 mM Cl<sup>-</sup> (**Table I**). Since the inhibition is partly reversed by the addition of bicarbonate, this result indicates that this treatment removes bicarbonate to only a certain extent from its binding site at PSII. The critical result, however, was that we found a substantial release of CO<sub>2</sub> by the addition of formate to NaCl-treated thylakoids of not only peas (**Fig. 5**), but also of maize (**Fig. 6**). Both the reaction medium and the boiled thylakoid controls did

Table I. The rate of oxygen evolution by maize and pea thylakoids after incubation in the dark at 20 °C for 30 min in a CO<sub>2</sub>-free medium containing 50 mM sodium phosphate and 10 mM NaCl at pH 6.4. Then, after collection by centrifugation and resuspension of the thylakoids in fresh CO<sub>2</sub>-depleted reaction medium to 50 µg Chl.ml<sup>-1</sup>, 10 mM bicarbonate was added and, after a further 2 min dark incubation, the electron acceptor system was added and oxygen evolution was measured (A. "+ 10 mM NaCO<sub>3</sub>"). The B. "no addition" sample was treated in the same way, but no bicarbonate was added. A third sample (C. "+ 100 mM NaHCO<sub>2</sub>") was again treated in the same way, but CO<sub>2</sub>-free formate was added. Oxygen evolution in µmol O<sub>2</sub>.mg Chl<sup>-1</sup>.h<sup>-1</sup>. Each number is the average of five experiments. The horizontal line D. "CO<sub>2</sub> release" indicates the number of CO<sub>2</sub> released per PSII reaction centers upon the addition of 100 mM formate to sample B, as measured according to Figs 5 and 6 (numbers are average of 4 experiments).

Additions	Maize thylakoids	Pea thylakoids
A. + 10 mM NaHCO <sub>3</sub>	198 ± 13	177 ± 11
B. no addition	178 ± 14	153 ± 14
Percent inhibition	10 ± 2	13 ± 3
C. + 100 mM NaHCO <sub>2</sub>	61 ± 6	37 ± 7
Percent inhibition	69 ± 3	79 ± 3
D. CO <sub>2</sub> release/PSII	0.54 ± 0.04	1.04 ± 0.10

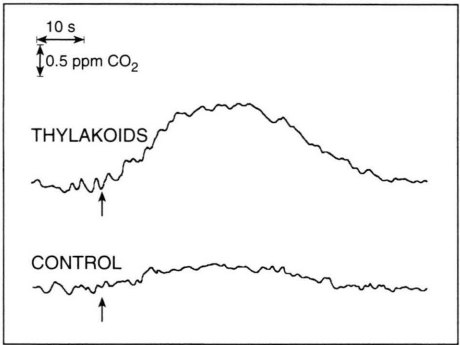


Fig. 5. CO<sub>2</sub> release by pea thylakoids after injection of 100 mM formate. At the time of the upward arrow, 0.3 ml gas sample was injected in the IRGA, taken from the gas phase above a thylakoid suspension which was incubated with 100 mM formate during 15 min. The control recording is from an experiment in which the same medium was used without thylakoids. One representative result out of four experiments.

not show any CO<sub>2</sub> release (data not shown). This means that, although the thylakoids were incubated in CO<sub>2</sub>-free medium for 30 min, they still have bicarbonate bound at their binding site which may be removed by the addition of formate. We speculate that a linear response of the injected and the measured CO<sub>2</sub>, the absence of any CO<sub>2</sub> leak

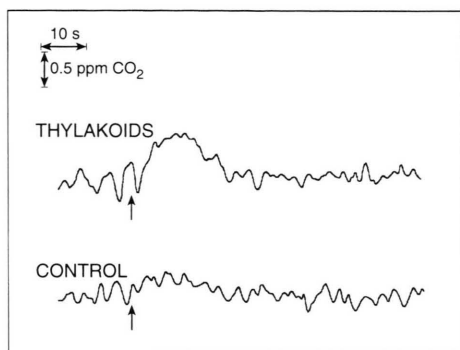


Fig. 6.  $\text{CO}_2$  release by maize thylakoids after injection of 100 mM formate. Same protocol as in Fig. 5. One representative result out of four experiments.

in our system, the sensitivity of the infrared gas analyzer and the absence of any significant dark- $\text{CO}_2$  uptake by our thylakoid samples are perhaps some of the factors responsible for the difference in our study from that of Jursinic and Stemler (1992).

On the basis of the results presented above, we have calculated the number of  $\text{CO}_2$  released by the addition of 100 mM formate. Using the method of Lee and Whitmarsh (1989), we have measured the number of PSII reaction centers to total Chl present to be 1.6 mmol per mol Chl in peas grown at  $220 \mu\text{E m}^{-2} \text{s}^{-1}$ . The number of 1.6 mmol PSII reaction centers per mol total Chl means that there is 1 PSII reaction center per 600 Chl. The number of  $\text{CO}_2$  released, upon the addition of 100 mM formate, was within 10% error,  $\sim 1.0 \text{ CO}_2$  per PSII reaction center for peas (Table I). In maize, as grown here, the number of Chl molecules per PSII reaction center was also  $\sim 600$ . Thus, the amount of  $\text{CO}_2$  released per PSII reaction center in maize was, within 10% error, about 0.6 (Table I). If we assume that the incomplete inhibition, 70% and 80%, respectively (Table I), in our maize and pea samples, is because some bound  $\text{CO}_2$  was not removed here, we get the upper figure of 0.8 and  $1.25 \text{ CO}_2/\text{PSII}$  reactions. First, the different number of  $\text{CO}_2$  released per PSII reaction center may have physiological significance since there are differences in the amino acid sequence of the D1 protein of maize and peas, and the D1 protein has been suggested to be a site of

bicarbonate binding (see e.g., Govindjee and van Rensen, 1993; Govindjee, 1993). Second, we are still short of about  $1 \text{ CO}_2/\text{PSII}$  reaction center, as expected from  $2 \text{ CO}_2/\text{PSII}$  reaction center (Blubaugh and Govindjee, 1988a).

### Concluding Remarks

In conclusion, there is no more controversy whether there is an effect of bicarbonate on the donor or the acceptor side of photosystem II; it is on both sides (Figs. 3 and 4). Further, there is no more controversy whether bicarbonate is required or not for PSII. Clearly, bicarbonate is bound to photosystem II *in vivo*. In both maize and pea thylakoid suspensions depleted of  $\text{CO}_2$ , without any inhibitor, high rates of electron flow are correlated with the presence of calculated 0.8 to  $1.25 \text{ CO}_2$  per PSII reaction center (Figs. 5 and 6). Although these data qualitatively support the essential activator model for bicarbonate, it is clear that a maximum of 40 to 60% of the expected bicarbonate was observed. There are two possible explanations for the missing bicarbonate. First, with our method, not all bicarbonate may be released by formate addition, or detected by the IRGA measurements. Second, water molecules have been suggested to be involved in proton transport (see Scheiner, 1994), and, a chain of water molecules leading to the  $\text{Q}_\text{B}$  binding site has been shown to exist in bacterial reaction center (Ermler *et al.*, 1994) where no bicarbonate effect exists (Shopes *et al.*, 1989; Wang *et al.*, 1992). We speculate that both bicarbonate and water molecules may play a role in protonation reactions that also involve several key amino acids in PSII (for a detailed model, see Xiong *et al.*, 1996). We are of the opinion that the bicarbonate-reversible formate inhibition site, studied here and elsewhere, is related directly to the normal  $\text{CO}_2$  binding site, but the proof awaits further experimentation and analysis.

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