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*Regular paper*

# Bicarbonate effects in leaf discs from spinach

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*Key words:* Bicarbonate-reversible formate effect, $CO_2$ fixation, chlorophyll *a* fluorescence induction, electron transport, oxygen evolution, Photosystem II, plastoquinone electron acceptor, (spinach leaf discs)

## Abstract

In this paper, we show the unique role of bicarbonate ion in stimulating the electron transfer of photosystem II (PS II) in formate-treated leaf discs from spinach. This is referred to as the "bicarbonate effect" and is independent of the role of $CO_2$ in $CO_2$ fixation. It is shown to have two sites of action:

(1) the first, described here for the first time, stimulates the electron flow between the hydroxylamine donation site ("Z" or "D") and $Q_A$, the first plastoquinone electron acceptor and
(2) the other accelerates the electron flow beyond $Q_A$, perhaps at the $Q_AQ_B$ complex, where $Q_B$ is the second plastoquinone electron acceptor.

The first site of inhibition by formate-treatment is detected by the decrease of the rate of oxygen evolution and the simultaneous quenching of the variable chlorophyll *a* (Chl *a*) fluorescence of leaf discs infiltrated with 100 mM formate for about 10 s followed by storage for 10 min in dark. This is referred to as short-term formate treatment. Addition of bicarbonate reverses this short-term formate effect and restores fully both Chl *a* fluorescence and oxygen evolution rate. Reversible quenching of variable Chl *a* fluorescence of heated and short-term formate treated leaf discs, in the presence of hydroxylamine as an artificial electron donor to PS II, is also observed. This suggests that the first site of action of the anion effect is indeed between the site of donation of hydroxylamine to PS II (i.e. "Z" or "D") and $Q_A$. The second site of the effect, where bicarbonate depletion has its most dramatic effect, as well known in thylakoids, is shown by an increase of Chl *a* fluorescence of leaf discs infiltrated with 100 mM formate for about 10 min followed by storage for 10 min in dark. This is referred to as the long-term formate treatment. Addition of bicarbonate fully restores the variable Chl *a* fluorescence of these leaf discs. Chl *a* fluorescence transient of DCMU-infiltrated (10 min) leaf discs is similar to that of long-term formate-treated one suggesting that the absence of bicarbonate, like the presence of DCMU, inhibits the electron flow beyond $Q_A$.

*Abbreviations:* DCMU – 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea; $F_0$, $F_{max}$ – original, and maximum Chl *a* fluorescence levels after onset of illumination; $NH_2OH$ – hydroxylamine; pheo – pheophytin; PS II – photosystem II; PS I – photosystem I; $Q_A$ – first plastoquinone electron-acceptor of PS II; $Q_B$ – second plastoquinone electron-acceptor of PS II; Z – electron donor to PS II reaction center

## Introduction

The phenomenon of bicarbonate ($HCO_3^-$) dependence of electron flow in photosynthesis has been known since the original work of Warburg and Krippahl (1958, 1960). An important element in deciphering the mechanism of this phenomenon in *in vitro* preparations was the use

of anions such as formate that accentuated the inhibition of electron flow due, we believe, to the further removal of $HCO_3^-$ from their binding sites (Good 1963, Stemler and Govindjee, 1973, Blubaugh and Govindjee 1988a). Bicarbonate has been unique in fully reversing the effect of inhibitory anions. We shall refer to this stimulatory effect as the "bicarbonate effect" in this paper. A major $HCO_3^-$ effect has been shown, in thylakoid membranes, to be located on the electron acceptor side of photosystem II (PS II) between $Q_A$, the first plastoquinone acceptor, and the plastoquinone (PQ) pool (Wydrzynski et al. 1975, Govindjee et al. 1976, Jursinic et al. 1976, Siggel et al. 1977, Vermaas and Govindjee 1982, Govindjee and Eaton-Rye 1986). However, the current knowledge about the existence of this $HCO_3^-$ effect *in vivo* is scarce. A $HCO_3^-$ effect in intact algal cells was suggested to exist in *Chlamydobotrys* (Wiessner et al. 1981) and *Scenedesmus* (Govindjee and Eaton-Rye 1986). Mende and Wiessner (1985) concluded that $HCO_3^-$ depletion affects both the oxygen-evolving side and the electron acceptor side of PS II in algal cells. Based on the measurements of flash-induced electrochromic absorbance at 515 nm (A 515) and chlorophyll *a* (Chl *a*) fluorescence, a role of $CO_2$ ($HCO_3^-$) in the regulation of the electron flow at the acceptor side of PS II was suggested to exist in non-formate treated leaves (Garab et al. 1983). Chl *a* fluorescence data in leaves, under conditions of very low photosynthesis, have suggested that $CO_2$ ($HCO_3^-$) may have a physiological role in the regulation of plastoquinone reactions (Ireland et al. 1987). Thermoluminescence measurements in non-formate treated leaves have shown that the charge accumulation in PS II is affected by $CO_2$ ($HCO_3^-$) suggesting its role in controlling the electron flow from PS II to PS I (Garab et al. 1988).

The current investigation was undertaken to exploit the use of formate, as used earlier in thylakoid membrane studies, to accentuate the bicarbonate effect in leaves in order to study its mechanism *in vivo*. Based on Chl *a* fluorescence and $O_2$ evolution measurements, we show here the existence of two sites of bicarbonate effect in spinach leaf discs, one before and the other after $Q_A$. The first site of action, that was detected by short (about 10 s) infiltration followed by 10 min storage in dark in formate medium, is suggested to be located between the electron donor "Z" (or "D") and $Q_A$ since it was present even when hydroxylamine was used as an artificial electron donor. The other site, that was detected by long (10 min) infiltration followed by 10 min storage in dark in formate medium, is located beyond $Q_A$. Preliminary abstracts for work on *Chlamydomonas* cells and spinach leaf discs were presented earlier (El-Shintinawy and Govindjee 1989a,b).

## Materials and methods

### *Treatments*

Spinach leaves were obtained from local market. Leaf discs, of 3.5 cm diameter, were depleted of $CO_2$ by the infiltration method (Garab et al. 1983). The idea of using the infiltration technique was to replace air in the intercellular space with the media we had used in our earlier thylakoid work (Eaton-Rye and Govindjee 1984, Blubaugh and Govindjee 1986). This was done to allow comparison with older data on thylakoids and to accentuate the bicarbonate effect due to the use of formate in the media. Furthermore, the use of infiltration method allowed us to obtain information on the $HCO_3^-$ effect in the presence of low or no $CO_2$ fixation: infiltration with aqueous medium is known to decrease or stop $CO_2$ fixation. We realize that the infiltration process itself has some effects on the fluorescence measurements but since comparison is always made with and without $HCO_3^-$ samples, both being infiltrated, the effect of infiltration does not affect our conclusions. The $CO_2$-depletion–infiltration medium (pH 5.8) contained: 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM sodium phosphate and 100 mM sodium formate (unless otherwise stated). Low pH was chosen as it has also been shown to accentuate the bicarbonate effect (Stemler and Govindjee 1973). The short-term formate treatment involved infiltration for 10 sec followed by storage for 10 min in dark before measurements began. On the other hand, long-term formate treatment involved infiltration for

10 min followed by storage for 10 min in dark before measurements began. Different concentrations of bicarbonate were introduced to the depletion–infiltration medium and then the pH was readjusted back to 5.8. Heating and hydroxylamine treatments were carried out by first immersing the leaf discs in a beaker containing distilled water for 3 min at 45°C, then they were left to cool at room temperature in a petri dish, and finally they were immersed in 10 ml infiltration media containing 15 mM $NH_2OH$ at pH 7.2 and infiltrated for 10 s under vacuum. Formate-treatment was carried out by immersing the leaf disc in a 10 ml formate-containing depletion medium in a stoppered Dewar flask which was used in fluorescence measurements. The leaves were infiltrated for the desired time under vacuum and then exposed to $CO_2$-free air. Formate was not removed from the sample during further storage and measurements. All samples were dark-adapted for 10 min before fluorescence measurements.

*Simultaneous measurements of oxygen evolution and Chl a flourescence*

These measurements were made by a Walker instrument (Hansatech Ltd., U.K.) which simultaneously measures oxygen evolution and Chl *a* fluorescence from leaf discs. For a discussion of Chl *a* fluorescence and interpretations, see Briantais et al. (1986) and Govindjee et al. (1986). The electrode was calibrated and the $O_2$ exchange calculated at 25°C according to Thomas et al. (1983). Samples were illuminated with blue light obtained by passing white light through two Corning glass filters (CS4-71 and CS5-56). The light source was a quartz halogen lamp giving a photon flux density at the leaf surface of 200 $\mu$mol $m^{-1}$ $s^{-1}$. The rate of oxygen evolution and fluorescence transient were measured in control (untreated) as well as in the treated leaf disc.

*Detailed measurements on Chl a fluorescence transient*

A separate fluorescence measurement was necessary because the Walker instrument, used above, does not allow precise measurements of the "O" level, necessary for calculating the variable Chl *a* fluorescence related to photochemistry and the reduction of $Q_A$ and for the further analysis of fluorescence data. In this study, "O" level was determined by two independent methods:

(a) by extrapolation of fluorescence rise to zero time; and
(b) by calculating from low intensity measurements.

Both gave results within 5–10% of each other. Chlorophyll *a* fluorescence transient measurements were carried out using a laboratory-built spectrofluorometer (Blubaugh 1987). The leaf discs were illuminated by a Kodak 4200 slide projector. The high-intensity projector light was filtered by a 56% neutral density filter and two Corning blue glass filters CS4-71 and CS5-56 (the same filters used in the Walker instrument). The photon flux density at the leaf surface was 30 $\mu$mol $m^{-2}$ $s^{-1}$. Chl *a* fluorescence emitted from the leaf discs was measured at 685 nm (3.3 nm slitwidth) by a S-20 photomultiplier (EMI9558B) through a Bausch and Lomb monochromator, protected by two complementary red Corning filters (CS2-61). A Biomation 805 waveform recorder and an LSI-11 Computer were used to store and analyze the fluorescence signals.

## Results and discussion

*Effect of time of infiltration with formate-containing buffer*

Figure 1 shows the effect of infiltration time in parallel measurements with the Walker instrument on both Chl *a* fluorescence (measured at the peak "P" that falls at 1 s of illumination here) and the rate of $O_2$ evolution. After 15 s infiltration in formate media (pH 5.8), the rate of oxygen evolution of the uninfiltrated leaf disc was decreased to 50% level of the original value of 28 $\mu$mol $O_2$ $m^{-2}$ $s^{-1}$ and the Chl *a* fluorescence intensity dropped to its minimum value as was observed in *Chlamydobotrys* (Mende and Wiessner 1985). Infiltrating the leaf discs for several minutes in the same media enhanced Chl *a* fluorescence gradually, but not the $O_2$ evolution rate.

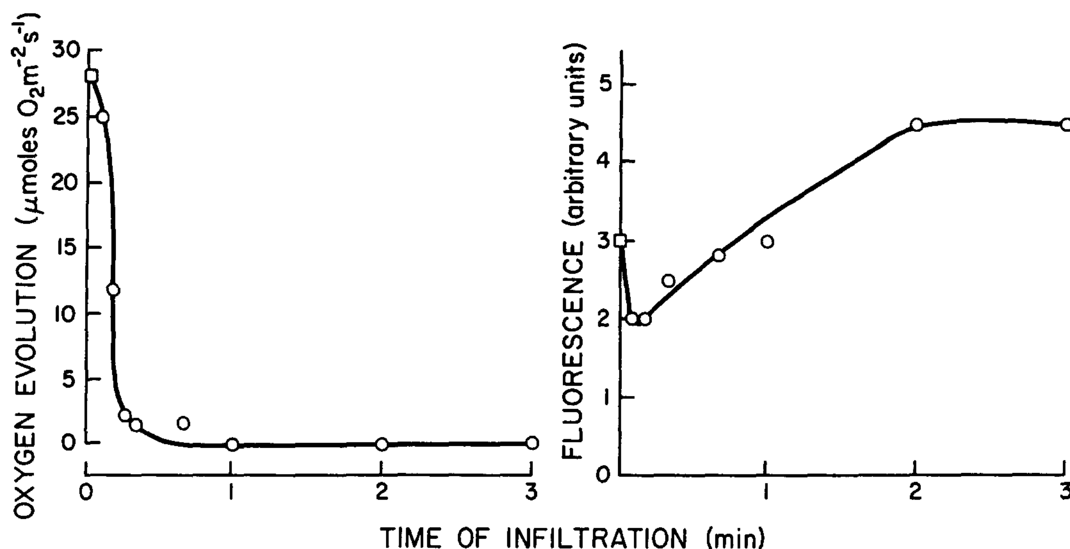


*Fig. 1.* The effect of infiltration time on simultaneous measurements of oxygen evolution rate (left) and Chl *a* fluorescence intensity after 1 s of illumination (P level) (right). One leaf disc was infiltrated under vacuum, in a 10 ml medium containing: 300 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM sodium phosphate and 100 mM sodium formate at pH 5.8. The open squares are for the control sample, uninfiltrated leaf disc, and the open circles are for treated samples. Incident intensity, 200 $\mu$moles $m^{-2}$ $s^{-1}$.

The decrease and the increase in Chl *a* fluorescence level, observed here, suggest the existence of two sites of formate-treatment effect. The decrease and increase in the fluorescence level was shown not to be due to change in the "O" level, but due to a change in the rate of O to P rise as measured for 15 s and 12 min infiltration points (see e.g., Fig. 4 at a later section; data not shown). Such a decrease and an increase in the variable fluorescence are usually interpreted as due to a block before and after the electron acceptor $Q_A$ (see e.g., Duysens and Sweers 1963). Chlorophyll *a* fluorescence intensity (F) is directly related to the rate constants ($k_s$) of various deexcitation pathways (fluorescence ($f$), heat loss ($h$), energy transfer to non fluorescent centers ($t$) and photochemistry ($p$)), number of absorbed photons ($I_{abs}$) and the concentration of open reaction centers $[Q_A]$ as follows:

$$F = \frac{k_f}{k_f + k_h + k_t + k_p\,[Q_A]}\,(I_{abs})\,.$$

In our experiments, the intensity of exciting light, the percent of absorption and the absorption spectra remained unchanged (data not shown) suggesting that $I_{abs}$ and $k_f$ were constants. Changes in emission spectra at 77 K reflect changes in excitation energy from PS II to PS I and, by implication, changes in $k_t$ at room temperature from strongly fluorescent PS II to weakly fluorescent PS I (see e.g., Briantais et al. 1986). Our measurements of formate/bicarbonate effects on 77 K emission spectra of thylakoids from leaves (Govindjee 1977) and of leaves (unpublished data) remain unchanged upon formate treatment suggesting that $k_t$ remains constant. Furthermore, during the fast fluorescence transient (O P phase), no evidence exists for changes in $k_h$ (see e.g., Briantais et al. 1986). Thus, since we observe a decrease in electron flow (reduced $O_2$ evolution) during short-term formate treatment, we can infer that decreased fluorescence is due to decreased electron flow to $Q_A$.

The drastic decrease in $O_2$ evolution is due to a combination of a block in the rate of electron flow by formate and a decrease in $CO_2$ fixation due to the infiltration process itself (see e.g., Fig. 3 at a later section). Since the Chl *a* fluorescence intensity increases when no change is observed in the net rate of $O_2$ evolution as the infiltration time is increased from 30 s to 3 min, the former change cannot be related to decreases in $CO_2$ fixation. Thus, it appears that increases in Chl *a*

fluorescence are independent of $CO_2$ fixation, as also suggested by Ireland et al. (1987).

It should be mentioned that the response time, shown in Fig. 1, differed slightly from leaf to leaf and depended also on the formate concentration used in the infiltration media and its pH. In alkaline media (pH 7.5) containing low formate concentration more time was needed to produce the same effects, as observed above (data not shown).

*Two sites of formate/bicarbonate action*

*(1) Short-term formate effect*

Table 1 shows the effects of "short-term formate treatment" on $O_2$ evolution and Chl *a* fluorescence transient. Average of 5 different experients is presented. When the leaf disc was infiltrated for 10 s in media containing no formate (pH 5.8), $O_2$ evolution rate of control sample (untreated leaf disc) dropped by about 40% (see Fig. 2, panels a and b). At the same time the Chl *a* fluorescence level decreased by about 20%. Some effect due to the infiltration process itself and, perhaps, due to a partial $CO_2$ depletion at pH 5.8 (even without formate) is expected. We show here that both the rate of $O_2$ evolution and the fluorescence level increased to within 90% of the control upon the addition of about 300 $\mu$M $HCO_3^-$ (Fig. 2, panel c). However, inclusion of 100 mM formate in the media leads to a decrease in the $O_2$ evolution rate of almost 60%; simultaneously, the Chl *a* fluorescence level diminished by 40% (Fig. 2, panel d). Thus, formate accentuates the effect, as expected. Inclusion of 1 and 10 mM $HCO_3^-$ in the infiltration medium (pH was readjusted back to 5.8), not only increased the Chl *a* fluorescence level to within 70% and 90% of the control, respectively, but also the oxygen evolution rate to within 60% and 90% of the control (Fig. 2, panels e and f). The strong inhibition by formate ion is obvious since the maximum quenching could be observed only in formate-infiltrated sample (panel d).

*(2) Long-term formate effect*

Table 2 shows the effect of "long term formate treatment" on $O_2$ evolution and Chl *a* fluorescence. Average of 3 different experiments is presented. Prolonged infiltration for 10 min in media containing different formate/bicarbonate concentrations (pH 5.8) decreased drastically the rate of $O_2$ evolution in all cases. At the same time long infiltration produced different effects on Chl *a* fluorescence depending on the content of the infiltration medium. The $O_2$ evolution rate of the control (uninfiltrated leaf disc) decreased by about 80% when the leaf disc was infiltrated in medium containing no formate (Fig. 3, panels a and b). At the same time, the Chl *a* fluorescence level, after 1 s of illumination, increased slightly (about 10%). Again, some effect due to infiltration process itself and, perhaps, due to a partial $CO_2$ depletion at pH 5.8, even without formate, is expected. Although, the presence of 35 $\mu$M $HCO_3^-$ (Fig. 3, panel c) in the infiltration medium did not lead to any change in the $O_2$

*Table 1.* Effects of "short term formate treatment" on $O_2$ evolution and Chl *a* fluorescence transient

| Treatment | $O_2$[a] | F[b] |
|---|---|---|
| (A) *In the absence of Formate*: | | |
| Control; uninfiltrated | 35 ± 3.4 | 100 ± 11 |
| Infiltrated in non-formate medium | 21 ± 2.4 | 83 ± 3.3 |
| Infiltrated in non-formate medium + 300 $\mu$M* $HCO_3^-$ | 30 ± 3.9 | 92 ± 11 |
| (B) *In the presence of Formate*: | | |
| Infiltrated in formate medium | 13 ± 2.4 | 58 ± 5.8 |
| Infiltrated in formate medium + 1 mM $HCO_3^-$ | 21 ± 2.9 | 67 ± 1.6 |
| Infiltrated in formate medium + 10 mM $HCO_3^-$ | 32 ± 3.7 | 92 ± 5.8 |

* Results with different concentrations in the 100–500 $\mu$M [$HCO_3^-$] range are averaged here.
[a] is expressed in $\mu$mol $O_2$ $m^{-2}$ $s^{-1}$.
[b] Fluorescence intensity, after 1 s of illumination (P level), is in arbitrary units.
Both the rates of $O_2$ evolution and fluorescence levels are given as averages with their standard deviations ($n = 5$). Incident intensity, 200 $\mu$mole $m^{-2}$ $s^{-1}$.

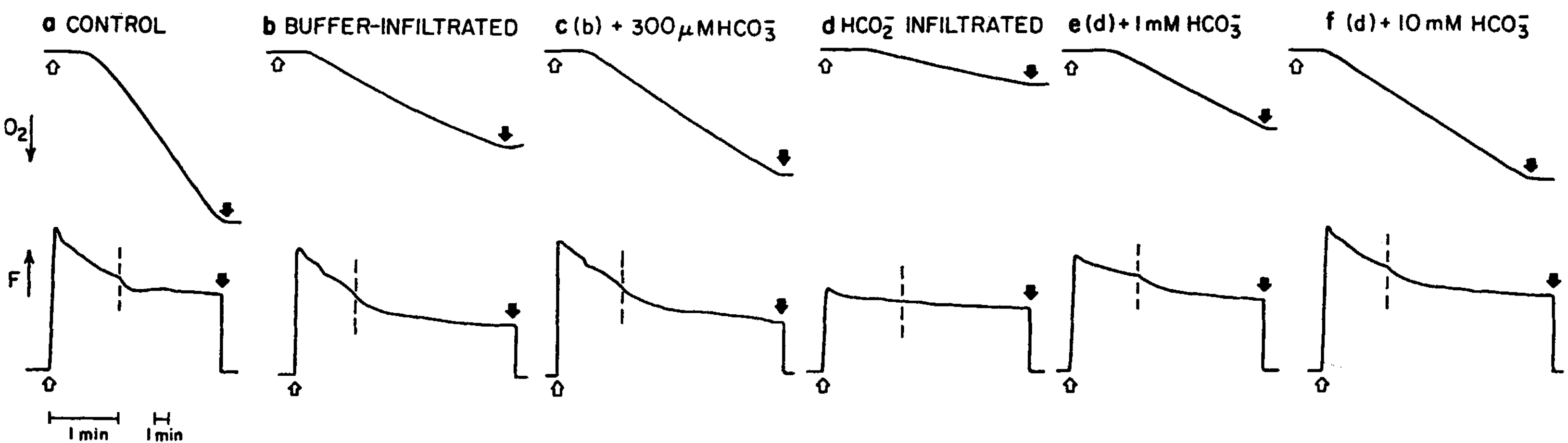


*Fig. 2.* The effect of short-term formate treatment on parallel measurements of the oxygen evolution rate ($O_2$) and Chl *a* fluorescence transient (F) of spinach leaf discs. Except for (a), the leaf discs were infiltrated for 10 s in the medium (pH 5.8) described in the legend of Fig. 1, but with conditions noted below. Samples were stored in dark for 10 min before measurements began. (a): $O_2$ and F for the control, untreated leaf disc. (b): the same for leaf disc infiltrated without formate. (c): as (b) + 300 $\mu$M $HCO_3^-$. (d): the same for leaf disc infiltrated with formate. (e): (d) + 1 mM $HCO_3^-$. (f): (d) + 10 mM $HCO_3^-$. Incident-intensity, 200 $\mu$moles $m^{-2} s^{-1}$.

*Table 2.* Effects of "long-term formate treatment" on $O_2$ evolution and Chl *a* fluorescence transient

| Treatment | $O_2$[a] | F[b] |
|---|---|---|
| (A) *In the absence of formate*: | | |
| Control; uninfiltrated | 35 ± 3.4 | 100 ± 10 |
| Infiltrated in non-formate medium | 6.7 ± 3.3 | 113 ± 5 |
| Infiltrated in non-formate medium + 35 μM $HCO_3^-$ | 6.7 ± 2.5 | 88 ± 7.5 |
| (B) *In the presence of formate*: | | |
| Infiltrated in formate medium | 9 ± 4.3 | 150 ± 6.3 |
| Infiltrated in formate medium + 4 mM $HCO_3^-$ | 8 ± 4.2 | 113 ± 2.5 |

[a] is expressed in μmol $O_2$ $m^{-2}$ $s^{-1}$.
[b] Fluorescence intensity, after 1 s of illumination (P level), is in arbitrary units.
Both the rates of $O_2$ evolution and fluorescence levels are given as averages with their standard deviations ($n = 3$). Incident intensity, 200 μmoles $m^{-2}$ $s^{-1}$.

evolution rate, Chl *a* fluorescence level was diminished (by about 20%). In 100 mM formate-infiltrated sample (Fig. 3, panel d) Chl *a* fluorescence level increased by about 50% while in 4 mM bicarbonate-restored case (Fig. 3, panel e) it decreased back to within 10% of the control, while $O_2$ evolution rate remained mostly unaffected. The fluorescence intensity at 1 s after illumination, that is close to the maximum Chl *a* fluorescence ($F_{max}$), after long time and 100 mM formate infiltration (pH 5.8), is similar to that produced by DCMU treatment. Higher pH and/or lower formate concentrations did not produce the real $F_{max}$. Infiltration in 10 μM DCMU produced $F_{max}$ that remained the same under all experimental conditions (data not shown). Here, the block in electron flow beyond $Q_A$ is total and the smaller blocks (i.e., slowing of reactions) elsewhere are no longer of any importance.

The above data (Figs 1, 2 and 3; Tables 1 and 2) suggest the existence of two bicarbonate-reversible formate effects in spinach leaf discs. We have further suggested that one of the two sites of action for affecting the electron flow in PS II is before and the other after $Q_A$. We now present data to further characterize these effects.

### *"Z" to $Q_A$ electron flow*

There are several possible explanations for the reversible quenching of Chl *a* fluorescence observed in Fig. 2, panel d as noted earlier. Since the fluorescence quenching is accompanied by a dramatic decrease of the oxygen evolution rate, the possibility of increased electron flow must be rejected. The alternative of the decreased electron flow to $Q_A$ for the quenching of Chl *a* fluorescence was examined by using hydroxylamine as an artificial electron donor to "Z" (or "D") in heat-treated samples, bypassing the $O_2$ evolving complex, but restoring variable Chl *a* fluorescence (see e.g., Mohanty et al. 1971). Experiments were done at pH 7.2 when hydroxylamine was used since this is the condition needed for hydroxylamine donation (for the details of hydroxylamine treatment, see *Materials and methods*); all other experiments were at pH 5.8. First, it is necessary to establish several controls. Figure 4A shows restoration of Chl *a* fluorescence transient upon the addition of 10 mM $HCO_3^-$ (trace 2 is identical to the control) to the short-term formate-treated disc (trace 1). Figure 4B (trace 2) shows the disappearance of most of the variable Chl *a* fluorescence in heated and buffer-infiltrated leaf disc as is known in thylakoids. Presence in 15 mM $NH_2OH$ in the infiltration medium (Fig. 4B, trace 3) restored, as expected, the variable Chl *a* fluoresence to that of the control leaf disc (trace 1) that was infiltrated in non-formate buffer. We realize that the pH for trace 3 was different from that for traces 1 and 2. Figure 4C however, shows the key data: here, the quenching of Chl *a* fluorescence of short-term formate treated leaf disc is present even when hydroxylamine, that donates electrons near "Z", is used (trace 2). Furthermore, inclusion of 10 mM $HCO_3^-$ in the formate medium fully restores Chl *a* fluorescence also in the presence of hydroxylamine (trace 3) to the control leaf disc that was infiltrated in buffer only without formate (trace 1). Since both of the

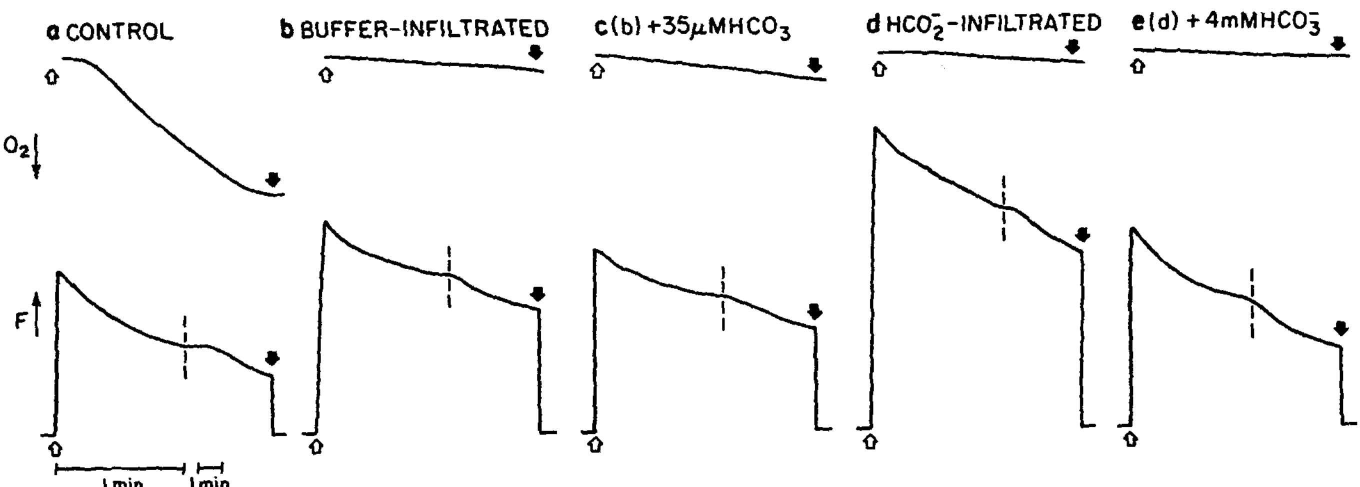


*Fig. 3.* The effect of long-term formate treatment on oxygen evolution rate and Chl *a* fluorescence of spinach leaf discs. Except for (a), the leaf discs were infiltrated for 10 min in the medium (pH 5.8) described in the legend of Fig. 1, but with conditions noted below. Samples were stored in dark for 10 min before measurements began. (a): $O_2$ and F for the control, untreated leaf disc. (b): the same for leaf disc infiltrated without formate. (c): (b) + 35 $\mu$M $HCO_3^-$. (d): the same for leaf disc infiltrated with formate. (e): (d) + 4 mM $HCO_3^-$. Incident intensity, 200 $\mu$moles $m^{-1}$ $s^{-1}$.

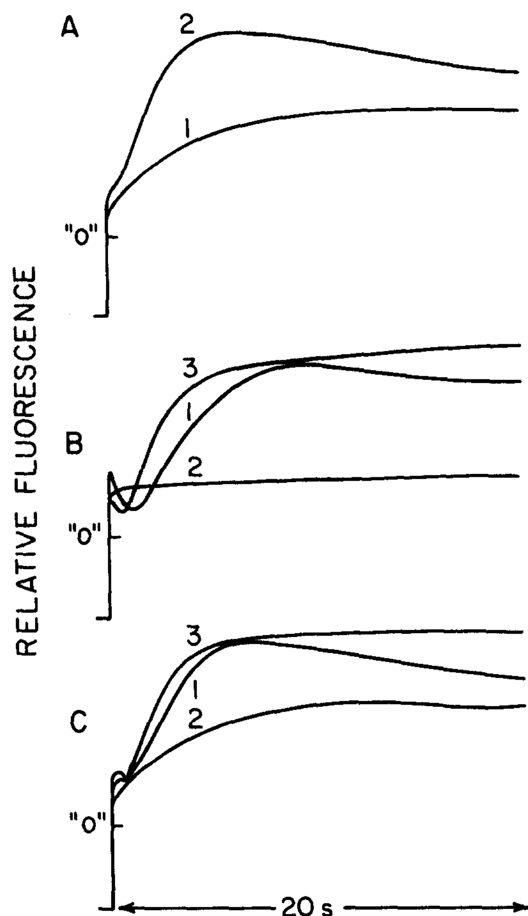


*Fig. 4.* Chl *a* fluorescence transient of leaf discs infiltrated for 10 s in different media. A 10 min storage in dark preceded all measurements. Whenever used, [$NH_2OH$] was 15 mM at pH 7.2; in all other cases, pH was 5.8. [$HCO_3^-$] was 10 mM and heating was at 45°C for 3 min. (A) Trace 1: leaf disc was infiltrated in a medium (pH 5.8) described in the legend of Fig. 1. Trace 2: same as 1 + 10 mM $HCO_3^-$. (B) Trace 1: control, leaf disc was infiltrated in a medium described in the legend of Fig. 1, but without formate. Trace 2: heat-treated leaf disc; the leaf disc was first heated then infiltrated in the same buffer as in trace 1. Trace 3: same as trace 2 + $NH_2OH$. (C) Trace 1: control, same as trace 1 in (B). Trace 2: leaf disc was first heated then infiltrated in formate medium containing $NH_2OH$. Trace 3: same as 2, +10 mM $HCO_3$. Incident intensity, 30 $\mu$moles $m^{-2}$ $s^{-1}$. The position of peak P is shifted to longer time (5 s) as compared to that in Figs. 2 and 3 (1 s) due to the use of lower light intensity here.

traces, 2 and 3, are at pH 7.2 the pH difference, mentioned above, is of no consequence to our conclusions. This shows that the site of inhibition by short term formate-treatment and, consequently, its reversibility by the inclusion of bicarbonate is after the site of electron donation by hydroxylamine i.e., it is between "Z" and $Q_A$.

Saygin et al. (1986) suggested that the reversible inhibition of the ns-reduction kinetics of $P_{680}^+$ depends on acetate treatment in PS II *Synechococcus* particles. The reduction time was slowed down to 160 $\mu$s in acetate-treated particles while it was 23 ns upon acetate removal and in control samples. The differences between the phenomena shown in this paper and that by Saygin et al. are as follows: (a) our data was related to $NH_2OH$ to $Q_A$ reaction while Saygin et al.'s data was related to $H_2O$ to $P_{680}^+$ reaction and (b) we recovered Chl *a* fluorescence by including low concentrations of $HCO_3^-$ in the 100 mM $HCO_2^-$ containing media, while Saygin et al. did not show restoration of their effect after $HCO_3^-$ addition. Bicarbonate, however, is known to have a small effect on the electron donor side of PS II (between $H_2O$ and "Z") by replacing $Cl^-$ (see e.g., a full discussion by Jursinic and Stemler 1988). We conclude that the reversible site of formate action observed in our study is different from that observed for acetate action since our effect of short-term treatment is on $NH_2OH$ to $Q_A$ reaction ($H_2O$ to "Z" reaction was intentionally stopped by heat treatment). Since the effect on the donor side ($H_2O$ to "Z") was not examined under short-term treatment, we are unable to comment on that site in this paper.

### *Inhibition of electron flow, by formate, beyond $Q_A$*

It has been suggested, although not proven, that formate replaces bicarbonate at its binding sites (Good 1963) and, thus, it is assumed that formate treatment causes bicarbonate depletion which inhibits the electron flow between $Q_A$ and $Q_B$, and beyond (Govindjee et al. 1976, Khanna et al. 1977, Siggel et al. 1977). Competitive binding between herbicides such as DCMU and quinone has also been observed in thylakoids

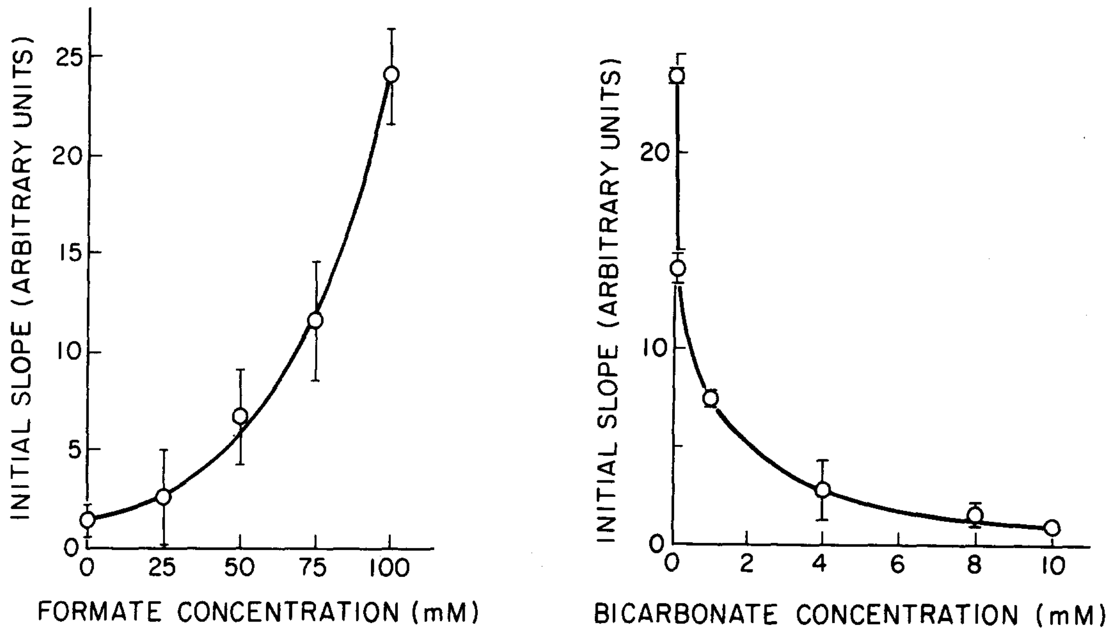


*Fig. 5.* Initial slope of Chl *a* fluorescence rise during 1 s after illumination. Average of at least 3 different measurements is shown. *Left*: as a function of formate concentration; the leaf discs were infiltrated for 10 min in the medium described in the legend of Fig. 1, but containing different [$HCO_2^-$] at pH 5.8. *Right*: as a function of bicarbonate concentration; the leaf discs were infiltrated for 10 min in the medium described in the legend of Fig. 1, but containing different [$HCO_3^-$] at pH 5.8.

(see e.g., Oettmeier and Soll 1983). There was a similarity between the Chl *a* fluorescence transient of 100 mM formate and 10 μM DCMU-treated leaf discs (data not shown). In both cases the maximum Chl *a* fluorescence ($F_{max}$) was reached quickly suggesting an almost complete blockage of electron flow at $Q_AQ_B$ complex. This result was in contrast to that from the sample infiltrated in the medium (pH 5.8) containing no formate. Here, Chl *a* fluorescence rose slowly. Dependence of the initial slope of the variable Chl *a* fluorescence upto 1 s after illumination, that reflects the reduction of the primary plastoquinone acceptor [$Q_A$] (Duysens and Sweers 1963, Govindjee et al. 1986), on formate concentration is shown in the left trace of Fig. 5. The initial sope of the Chl *a* fluorescence rise for 50 and 100 mM formate-treated sample is increased about 10 and 20 fold from that without formate suggesting that formate-treatment keeps the primary plastoquinone acceptor in a reduced state $Q_A^-$.

### *Reversible inhibition of electron flow, by bicarbonate, beyond $Q_A$*

The role of bicarbonate in reversing the formate effect on the electron flow beyond $Q_A$, the bicarbonate-reversible formate effect, is shown in the right trace of Fig. 5. The initial slope of Chl *a* fluorescence rise (upto 1 s after illumination) decreased by a factor of 2, 4 and 8, respectively, when 100 μM, 400 μM and 4 mM $HCO_3^-$ were included in the infiltration medium. It is comparable to the effects seen by Jursinic and Stemler (1986) in pea thylakoids. These results confirm the reversibilty of formate-treatment by added bicarbonate at the $Q_AQ_B$ complex in leaves. This stimulatory role of bicarbonate on electron flow is unique among all monovalent anions; formate inhibits photosystem II as in thylakoids (cf., Stemler and Jursinic 1983, Stemler and Murphy 1985). In our opinion, this inhibition is due to the ability of formate to remove $HCO_3^-$.

*Concluding remarks*

Our experiments establish the existence of large bicarbonate-reversible formate effect in a new system (spinach leaf discs). Although this system has many complications (such as variations among the leaves according to their age and the internal pH) we have found the same results by using a large number of leaf discs. Thus, these complications are of no significance to our conclusions. Blubaugh and Govindjee (1988a,b) suggested the existence of two binding sites in thylakoid membranes. We provide here evidence for the existence of two sites of action for the bicarbonate-reversible formate effect on the PS II Chl *a* fluorescence transients in leaf discs. It is not yet clear whether the suggested sites of anion binding are related to those studied here. The first site of action in our study is located between the electron donor "Z" and $Q_A$. This specific site has been identified here for the first time. We speculate that this site may be between pheophytin and $Q_A$. The other site of action, where bicarbonate has its most dramatic effect, is located beyond $Q_A$. Ireland et al. (1987) have suggested that this role of $CO_2$ is independent of its role in $CO_2$ fixation. We support this suggestion since Chl *a* fluorescence intensity was found to increase when there was no change in net $O_2$ evolution as the infiltration time was increased from 30 s to 3 min (Fig. 1).

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*Note added in proof*

A Stemler (Absence of a formate-induced release of bicarbonate from Photosystem II, Plant Physiol. **91**, 287–290, 1989) has recently reported that formate does not inhibit electron flow by removing bound bicarbonate, but it does so by binding to empty sides. This conclusion was based on the observation of the absence of a formate-induced release of $CO_2$ in maize thylakoids monitored by a mass spectrometer. In contrast, Govindjee, H.G. Weger, D.H. Turpin, J.J.S. van Rensen, O.J. de Vos and J.F.H. Snel (1989, unpublished observations) have shown, using a sensitive membrane inlet mass spectrometer and a sensitive differential $CO_2$ infrared gas analyzer, that formate treatment releases micromolar quantities of $CO_2$ from spinach and pea thylakoids: both the $CO_2$ release and the bicarbonate-reversible inhibition of electron flow are pH dependent and occur within minutes of formate treatment. These results are consistent with the hypothesis that native bound bicarbonate is required for electron flow in thylakoids.

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