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A MAJOR SITE OF BICARBONATE EFFECT IN SYSTEM II REACTION

EVIDENCE FROM ESR SIGNAL II_{vf}, FAST FLUORESCENCE YIELD CHANGES AND DELAYED LIGHT EMISSION

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SUMMARY

In order to determine the major site of bicarbonate action in the electron transport complex of Photosystem II, the following experimental techniques were used: electron spin resonance measurements of Signal II_{vf}, measurements of chlorophyll *a* fluorescence yield rise and decay kinetics, and delayed light emission decay. From data obtained using these experimental techniques the following conclusions were made: (1) absence of bicarbonate causes a reversible inactivation of up to 40 % of Photosystem II reaction center activity; (2) there is no significant effect of bicarbonate on electron flow from the charge accumulating S state to Z; (3) there is no significant effect of bicarbonate on electron flow from Z to $P\text{-}680^+$; (4) electron flow from Q^- to the intersystem electron transport pool is inhibited by from 4- to 6-fold under bicarbonate depletion conditions.

INTRODUCTION

The role of the bicarbonate anion in stimulating the Hill reaction has recently been investigated in our laboratory [1–5]. Addition of bicarbonate to samples previously depleted of bicarbonate causes as much as a 10-fold increase in the Hill reaction. Earlier attempts to locate the site (or sites) of bicarbonate action did not give an unequivocal answer. In this paper we show that a major site of bicarbonate action is on the reducing side of Photosystem II, that is, between Q (the primary electron acceptor of Photosystem II) and the intersystem electron carrier pool.

In this discussion the following flow diagram of the early steps in photosynthesis occurring at Photosystem II will be used:

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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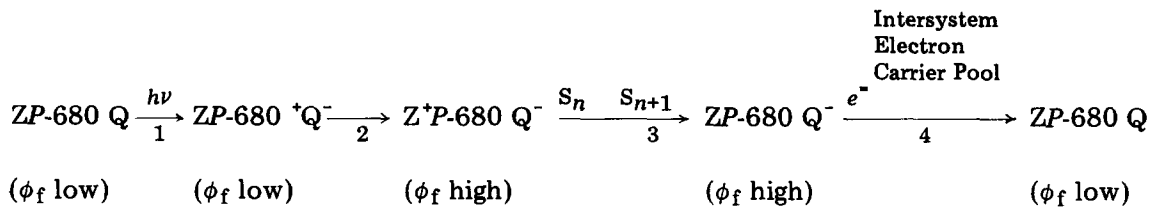

Reaction 1 is the initial charge separation which occurs in less than 20 ns [6]. Reaction 2 occurs when the first electron donor, Z, for Photosystem II reduces the oxidized reaction center II chlorophyll *a* *P*-680$^+$ [7]. The oxygen-evolving S states advance one step upon being oxidized by Z^+ in reaction 3 [8]. Finally, Q^- donates its electron to the intersystem electron carrier pool where it continues on its flow through the photosynthetic electron transport chain [9]. The bicarbonate effect at these various reaction sites has been tested using various techniques: reaction 2 by fluorescence yield rise, reaction 3 by ESR Signal II_{vf}, and reaction 4 by fluorescence yield decay and delayed light emission.

Using repetitive flash illumination, a rapidly decaying ESR signal referred to as Signal II_{vf} has been observed [10]. This ESR Signal II_{vf} has been attributed to Z^+ [10–12] and has been used to investigate the oxidation kinetics of S states of the oxygen-evolving system, reaction 3 in the above scheme.

The rapid rise in chlorophyll *a* fluorescence yield in the microsecond time range after a saturating flash indicates the electron transfer from Z to *P*-680$^+$ [7, 13–15]. Measurements of the rise in fluorescence yield were used to analyze the kinetics of reaction 2 above. The decay in fluorescence yield was used to measure reaction 4, as was previously shown [16–18].

The effect of bicarbonate upon delayed light in the microsecond time range is also observed. In this time range according to the recombination model of Lavorel [19, 20] delayed light reflects the decay of *P*-680$^+$ and Q^- and gives information about reactions 1, 2 and 4.

The results obtained together with experimental data kindly supplied by other groups (Diner, B., Döring, G. and Sinclair, J.) are consistent with an effect of bicarbonate depletion which: (1) causes a decrease in the number of active System II reaction centers, (2) slows the flow of electrons from Q^- to the intersystem carriers by up to 5-fold.

MATERIALS AND METHODS

For the ESR experiments, broken spinach chloroplasts (*Spinacea oleracea*) were used and prepared as previously described [10]. Sample chlorophyll concentrations were 3–4 mg/ml. In the chlorophyll *a* fluorescence yield and delayed light emission measurements, broken chloroplasts of maize (*Zea mays*) and romain lettuce (*Lactuca sativa*) were used. They were prepared according to the procedure of Stemler and Govindjee [1]. The sample concentration was adjusted to give an absorbance of 0.1 at 680 nm for a 1 cm pathlength. For both types of experiments chloroplasts were depleted of bound bicarbonate by the method of Stemler and Govindjee [1] as modified by Wydrzynski and Govindjee [5]. For ESR experiments a reaction mixture

consisting of 50 mM HEPES (pH 7.6), 1 mM NH_4Cl, 5 mM $MgCl_2$, 20 mM NaCl, 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$ or 10 mM NaCl, 100 mM sodium formate and 50 mM sodium phosphate buffer, pH 6.8, was used. For chlorophyll *a* fluorescence and delayed light emission measurements, the phosphate buffer at pH 6.8 was used. The bicarbonate depletion procedure was verified by measuring oxygen-evolving capacity in continuous light. 4–8-fold bicarbonate enhancement effects were routinely observed. Measurement of the chloroplast suspension pH upon addition of bicarbonate showed no change indicating that the observed effects were not due to changes in pH.

Flash photolysis ESR experiments were performed as described by Blankenship et al. [10]. The experimental arrangement used for making fluorescence yield rise measurements is shown in Fig. 1. Excitation light flashes were provided by a General Radio Strobotac 1538-A through two Corning C.S. 4-96 filters providing excitation in the Soret band of chlorophyll *a*. The pulse width at half height for the strobe was approx. 5 μs with a weak but extended tail. An EMI 9558B photomultiplier with S-20 cathode (protected by neutral density filters, a C.S. 2-64 and Schott RG-8 filter combination) was used for detection of fluorescence. The photomultiplier output circuit was designed to have a rise time of 400 ns. The signals were displayed on a Tektronix 551 oscilloscope with a 1A1 plug in amplifier and traces were photographed for later analysis. Flash lamp and oscilloscope triggering signals were provided by the Tektronix type 160 series pulse and waveform generators.

The fluorescence yield decay and delayed light emission measurements were made with the same experimental arrangement (Fig. 1) with the following changes in equipment: The actinic light source used was an AVCO Everett Model C102 nitrogen laser having an emission at 337 nm with a pulse width of 10 ns and virtually

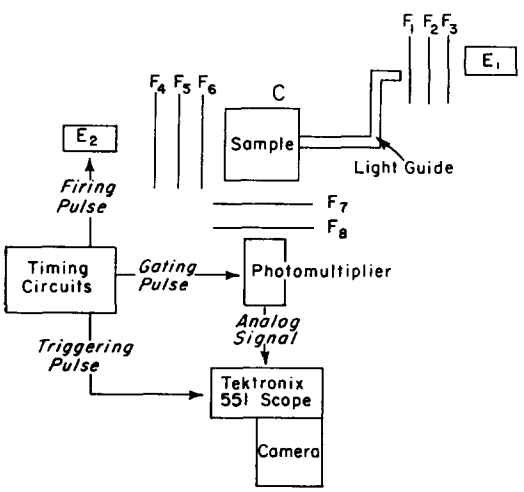

Fig. 1. Experimental arrangement for measuring the fluorescence yield rise and decay and delayed light emission (DLE). Symbols: C, quartz cuvette, 1 cm square; E_1, weak analytic flash, strobe light; E_2, main excitation flash; strobe light for fluorescence yield rise and nitrogen laser for fluorescence yield decay and delayed light emission measurements. F_1 and F_4, neutral density filters; F_2, F_3, F_5, and F_6, Corning C.S. 4-96 filters; F_7, Kodak Wratten 2-A (used only with nitrogen laser); F_8, Schott RG-8.

no tail. Fluorescence and delayed light emission were observed through a Kodak Wratten 2A and Schott RG-8 filter combination. The EMI photomultiplier was used; however, it was gated off electronically during the actinic light pulse. When making fluorescence yield decay measurements, a variable delay analytic flash was provided by another strobe lamp through neutral density filters, two C.S. 4-96 filters and a light guide.

The protocol used in determining the fluorescence yield rise was similar to that of Den Haan et al. [7]. Fluorescence ($F(\tau)$) and excitation light signals ($I(\tau)$) were photographed from the oscilloscope screen and the relative fluorescence yield (ϕ_f) was calculated as follows: $\phi_f = F(\tau)/I(\tau)$.

Fluorescence yield decays were measured by a method similar to that of Mauzerall [13]. The analytic flash was kept very weak so as to not cause a change in fluorescence yield when it was flashed onto the sample. The analytic flash generated fluorescence signals proportional to the yield of fluorescence at various time delays beginning 20 μs after the excitation flash. The fluorescence yield was calculated at a particular time after the main actinic flash using the formula:

$$\phi_f(\tau) \text{ (in terms of } \phi_0) = \frac{F_m(\tau) - I_{DLE}(\tau)}{F_0},$$

where $\phi_f(\tau)$ = fluorescence yield at some time τ after the main actinic pulse, $F_m(\tau)$ = measured signal at some time τ, $I_{DLE}(\tau)$ = intensity of delayed light emission at some time τ, F_0 = zero level fluorescence signal generated by pulsing the weak analytic flash without the main actinic flash, and ϕ_0 = zero level fluorescence yield.

RESULTS AND DISCUSSION

Bicarbonate effect on decay of ESR Signal II_{vf}

The ESR Signal II_{vf} is formed after a flash in less than 100 μs (the response time of the ESR spectrometer) and decays in dark with a half time of approx. 800 μs, independent of bicarbonate treatment (Fig. 2). However, bicarbonate depletion reduces the maximum amplitude of the ESR signal approx. 40 % compared to the control sample. The logarithmic plot of the Signal II_{vf} decay data shows that the half time ($\tau_{\frac{1}{2}}$) of decay is not significantly affected by bicarbonate depletion: 810 μs (with bicarbonate) versus 960 μs (without bicarbonate); this difference is within experimental error (Fig. 3). However, for this same sample preparation, oxygen evolution showed a 4-fold bicarbonate effect. The above experiments suggest that bicarbonate does not affect the rate of electron flow from the oxygen-evolving system "S" to Z, but the formation of Z^+ is reduced to some extent. This could either be due to a slightly reduced formation of $P\text{-}680^+$ or to a partial block of electron flow from Z to $P\text{-}680^+$. It is interesting to note that the 40 % reduction in Signal II_{vf} amplitude in bicarbonate-depleted sample is very close to the percentage of inactivated Photosystem II centers reported by Stemler et al. [4] from oxygen yield measurements.

It should be pointed out that even though ESR Signal II_{vf} gives information about the concentration of Z^+, the ESR detection technique has a limiting rise time of 100 μs and therefore is incapable of giving information about the formation of Z^+ and the consequent decay of $P\text{-}680^+$ in the early microsecond time range. Fortunately, the fluorescent yield technique does provide this information.

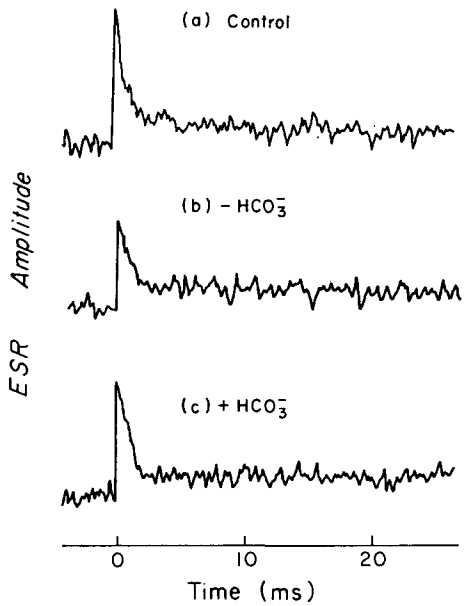

Fig. 2. Flash-induced change in ESR Signal II_{vf} in spinach chloroplasts at room temperature. a, untreated control chloroplasts; b, bicarbonate-depleted chloroplasts; c, bicarbonate-depleted chloroplasts plus 0.01 M $NaHCO_3$. Each trace is the average of 2048 flash events. Flash repetition rate, 1 Hz. The kinetic signal was monitored at the low field derivative maximum of Signal II (3380 G). Microwave power, 160 mW; modulation amplitude, 6.3 G.

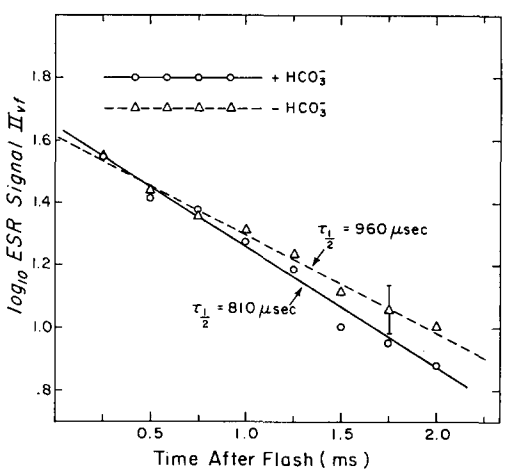

Fig. 3. Logarithmic plot of the ESR Signal II_{vf}. Data observed with and without bicarbonate. Calculated decay half times and a typical error bar are indicated.

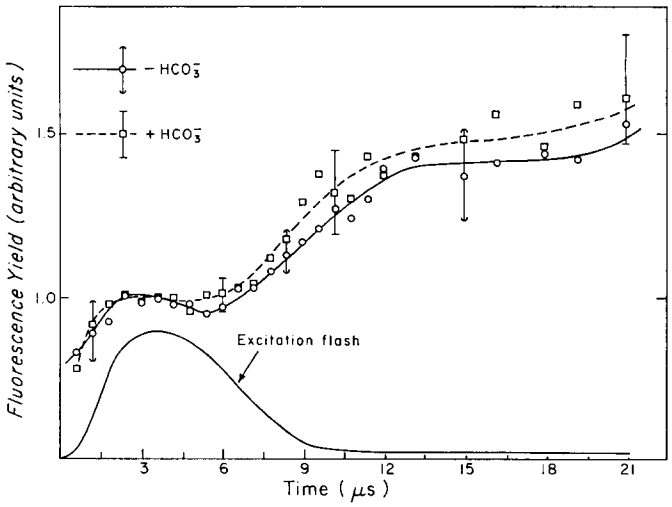

Fig. 4. The rise in fluorescence yield during and after an excitation flash with and without bicarbonate, normalized at $\tau = 3$ μs. A trace of the excitation flash intensity as a function of time is also shown.

Bicarbonate effect on the chlorophyll a fluorescence yield rise

The rise in fluorescence yield was measured beginning at about 1 μs and extending to 21 μs after the initiation of the excitation flash (Fig. 4). Each graphical point represents the mean value of seven experiments and typical error bars are shown. In this particular measuring method the rate of rise in the fluorescence yield up to about 3 μs is dependent upon the rapidity of the intensity rise in the excitation flash [14, 15]. The continuing rise in fluorescence yield beginning at about 6 μs shows two half times: 7 ± 3 μs and 35 ± 10 μs. No significant bicarbonate effect on the rise in fluorescence yield was observed. Since the rise in fluorescence yield in this time range is indicative of the rate of electron flow from Z to $P\text{-}680^+$, the above experiments suggest that the site of bicarbonate action is other than reaction 2 in the flow diagram. Thus, the 40 % reduction in ESR Signal II_{vf} (Z^+) observed under conditions of bicarbonate depletion must be due to a lower amount of $P\text{-}680^+$ being formed or a portion of the $P\text{-}680^+$ formed is rendered incapable of reacting with Z. The former interpretation will be supported by the delayed light emission results.

Bicarbonate effect on the fluorescence yield decay

A logarithmic plot of the fluorescence yield decay data from 50 μs to 1 ms after the illumination flash shows a 4.6-fold decrease in half time of decay when bicarbonate is added to a previously depleted sample (Fig. 5). Samples with maximum fluorescence yields of from 1.5 to 3 ϕ_0 were used and in all cases a bicarbonate-induced 3- to 5-fold increase in fluorescence yield decay rate was observed. For these same samples a 3–5-fold bicarbonate enhancement effect on oxygen evolution could be demonstrated. From the result it is concluded that a major site of bicarbonate action is between Q and the pool of electron flow intermediates, i.e. reaction 4 in the flow diagram. In point of fact, bicarbonate depletion acts as a partial block of electron flow between Q and the intersystem carriers in much the same way as DCMU does in

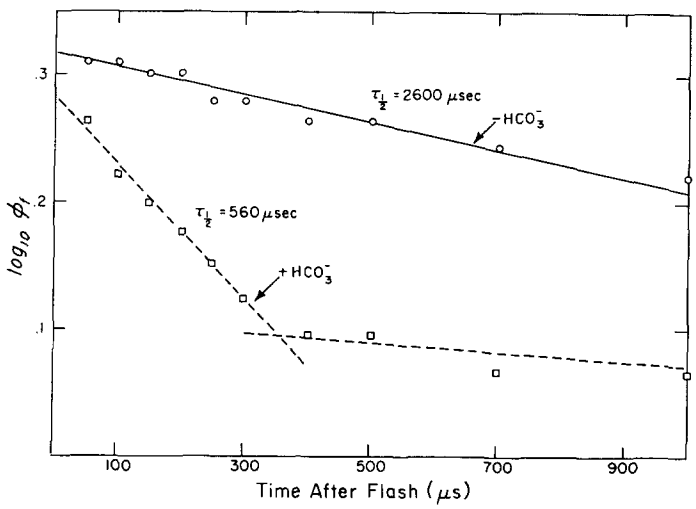

Fig. 5. Semilog plot of the decay of fluorescence yield with and without 10 mM bicarbonate.

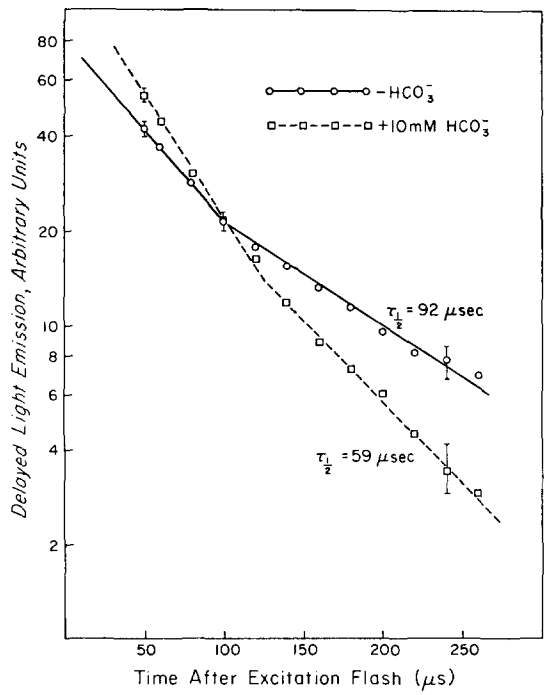

Fig. 6. Logarithmic plots of the delayed light emission signal traces with and without 10 mM bicar bonate. Calculated decay half times are indicated. Typical error bars are shown.

low concentrations. An analogous behavior of DCMU and low bicarbonate concentrate was previously pointed out by Wydrzynski and Govindjee [5].

The effect of bicarbonate depletion and DCMU and delayed light emission

Bicarbonate depletion resulted in two changes in the 50–250 μs delayed light emission decay (Fig. 6): A 1.5-fold reduction in the rate of decay and for $t < 100$ μs a decrease in delayed light emission intensity and for $t > 100$ μs a relative increase in delayed light emission intensity. In particular, at 50 μs after the flash, bicarbonate depletion caused approximately a 20 % reduction in delayed light emission. Sample with 10^{-8} and 10^{-7} M DCMU gave results similar to those obtained with the absence of bicarbonate: A 1.3–1.5-fold decrease in the decay rate for $t > 100$ μs and a 20–50 % reduction in delayed light emission intensity at 50 μs after the flash; but no significant increase in delayed light emission intensity for $t > 100$ μs was observed.

One theory to explain the origin of delayed light emission in the microsecond time range is that a back reaction takes place between P-680$^+$ and Q^-, the charge separation products of the Photosystem II light reaction [19, 20]. With a block of electron flow from Q^- to the pool of intersystem electron carriers the delayed light emission decay rate is expected to decrease since a competitive mode causing the reduction of the Q^- concentration is blocked. As can be seen in Fig. 6, the rate of delayed light emission decay is slowed under conditions of bicarbonate depletion. This result is in agreement with the concept of low bicarbonate acting as a block to electron flow between Q^- and the pool of intersystem electron carriers and that there is some inactivation of reaction centers. In addition, the reduction in delayed light emission for $t < 100$ μs in bicarbonate-depleted samples may be caused by a smaller amount of P-680$^+$ being formed which is consistent with the ESR Signal II_{vf} data presented. It is hard to compare this data to that of Stemler and Govindjee [2] since their samples were not given single flash illumination and the delayed light emission observations were only made for short lengths of time in the second time region, making kinetic analysis difficult.

Concluding remarks

Other unpublished results, obtained in other laboratories upon request by one of us (G.), support the view that the site of bicarbonate sensitivity is not on the oxygen evolving but on the Q side of Photosystem II. In one experiment, B. Diner (personal communication) has observed that the decay of Q^- measured by fluorescence decay is slowed down in the absence of bicarbonate anions. All of our seven experiments are in agreement with this data. G. Döring (personal communication) has observed the effects of bicarbonate concentration on the changes in P-680. Upon illumination by repetitive flashes, a decrease in absorption suggested to be due to oxidation of P-680 occurs (see review, ref. 21). In the dark these absorption changes decay as P-680$^+$ becomes reduced, thus giving a method of monitoring reaction 2 of the flow diagram. Döring found that the 120-μs decay component showed no significant change due to bicarbonate depletion or its later addition, but a 40 % reduction in the amplitude of P-680$^+$ was observed.

Using the modulated polarographic technique, first described by Joliot et al. [22], T. Arnason and J. Sinclair (personal communication) found that the rate constant associated with the rate-limiting thermal reaction between the water-

splitting reaction and Photosystem II was not altered in the absence of bicarbonate. Thus, it is clear that there are, at least, two sites of bicarbonate action, one on the reaction center complex II *P*-680 and the other between Q and the pool of intersystem electron carriers, the latter being a major site of action. This conclusion is additionally confirmed by the recent independent biochemical experiments of R. Khanna, T. Wydrzynski and Govindjee (personal communication) who show a major bicarbonate effect when plastoquinone is blocked by dibromothymoquinone and the electron acceptor is oxidized diaminodurene, but no significant effect when the electron acceptor is silicomolybdate which picks up electrons directly from Q or when electron flow is measured only through System I in the presence of DCMU (with reduced diaminodurene as electron donor and methyl viologen as electron acceptor).

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