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Bicarbonate or Carbon Dioxide as a Requirement for Efficient Electron Transport on the Acceptor Side of Photosystem II*

WIM F. J. VERMAAS
GOVINDJEE

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ABBREVIATIONS

B (or R)	Second quinone-type PSII electron acceptor
Chl	Chlorophyll
DAD	2,3,5,6-Tetramethylphenylenediamine
DBMIB	2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DCPIP	2,6-Dichlorophenolindophenol
DPC	Diphenylcarbazide
EPR	Electron paramagnetic resonance
FeCy	Ferricyanide [$Fe(CN)_6^{3-}$]
HCO_3^-*	Species (CO_2 or HCO_3^-) that binds to a specific binding site and allows efficient electron transport ($\equiv CO_2^*$ in Stemler, Chapter 15)

*This chapter should be read in conjunction with the chapter by A. Stemler—Editor.

Photosynthesis: Development, Carbon Metabolism, and Plant Productivity, Vol. II

ISBN: 0-12-294302-3

M	Charge accumulator in the oxygen-evolving system
MV	Methyl viologen
Pheo	Pheophytin
PQ	Plastoquinone
PSII	Photosystem II
Q	First quinone-type PSII electron acceptor
S	State of the oxygen-evolving system
SiMo	Silicomolybdate ($SiMo_{12}O_{40}^{4-}$)
$Z_{(1,2)}$	Donor(s) to P680

ABSTRACT

In this chapter, we reviewed observations showing that HCO_3^-* (CO_2 or HCO_3^-) greatly affects the electron transport rates between the quinone-type intermediates [Q, B, and the PQ (plastoquinone) pool] on the acceptor side of photosystem II (PSII) without directly influencing the donor side of PSII.* The major observations against an important role of HCO_3^-* on the donor (oxidizing) side and in favor of its role on the acceptor (reducing) side of PSII are

1. There is no HCO_3^-* effect on the electron transport from H_2O to Q as measured with silicomolybdate as electron acceptor in the presence of DCMU or with ferricyanide in trypsin-treated chloroplasts (Section II,A).
2. There is no HCO_3^-* effect on the kinetics of electron flow from the oxygen evolving system to the electron donor Z to P680, and from Z to P680 (Section II,E).
3. There is a large HCO_3^-* effect on electron transport from Q^- to B, as measured by both fluorescence and absorption methods (Sections II,B, and II,C); an additional, but smaller, effect is observed between P680 and Q.
4. The Chl *a* fluorescence transient in CO_2-depleted samples is similar, but not identical, to that in the presence of DCMU; the latter blocks electron flow beyond Q. Detailed analysis suggests that the absence of HCO_3^-* causes a major block after B (Section II). A large effect on the electron transport from B^{2-} to PQ is also confirmed by measurements of (a) Chl *a* fluorescence after single flashes of light; and (b) absorption changes at 265 nm (Sections II,B and C).
5. The location of the binding site of HCO_3^-* is close to that of the herbicides that bind near Q and B (Section II,F).

The bottle-neck reaction in CO_2-depleted chloroplasts seems to be electron transport from B^{2-}† to PQ, with a $t_{1/2}$ of at least 100 msec; in thoroughly depleted samples, this reaction takes seconds suggesting a "complete" block. After HCO_3^- addition, the PQH_2 oxidation ($t_{1/2} \sim 25$ msec) becomes rate-limiting, like in control chloroplasts. Most of the Q^- reoxidation by $B^{(-)}$ is faster than the PQ reduction in CO_2-depleted chloroplasts. No experimental data, thus far, point unequivocally to any CO_2/HCO_3^- effect on the donor side of PSII.

This chapter does not offer a complete picture of the HCO_3^- or CO_2 action in thylakoid membranes; it only deals with the site of action of the "bicarbonate effect" on photosynthetic electron transport. Therefore, in order to obtain an "overview" of this subject, this chapter should be read in conjunction with the cited reviews and with Chapter 15.

†In this chapter, the description "B^{2-}" is used to denote fully reduced B in general, and the use of "B^{2-}" does not exclude protonation of the reduced form of this secondary quinone.

I. Introduction

A crucial role of CO_2 or bicarbonate on the oxygen evolving mechanism of photosynthesis, as presented in Chapter 15 of this volume, is a point of view that is interesting but speculative inasmuch as it does not yet have a firm experimental backing. The present chapter is written to provide the reader with information on the action of CO_2 or HCO_3^- on the acceptor side of PSII, for which, contrary to a bicarbonate effect on the donor side of PSII, strong evidence exists. The absence of HCO_3^-* results in a severe slowing down of electron transport between Q, the first quinone-type PSII acceptor, and the PQ pool, without directly affecting electron transport on the donor side of PSII (see Fig. 1). This effect of HCO_3^-* has been established by many types of experiments in several laboratories (see, e.g., Wydrzynski and Govindjee, 1975; Govindjee *et al.*, 1976; Jursinic *et al.*, 1976; Khanna *et al.*, 1977, 1980, 1981; Siggel *et al.*, 1977; Stemler, 1977, 1979; van Rensen and Vermaas, 1981a; Vermaas and van Rensen, 1981). Some of these experiments will be summarized later. It is our hope that the reader after having read this and Chapter 15 may have a better appreciation of the complicated, but interesting and important, bicarbonate problem.

In addition to its effect on electron transport, bicarbonate has been shown to influence other photosynthetic processes in the thylakoid membrane, including enhancement of photophosphorylation at pH 7.0–7.5 (Punnett and Iyer, 1964). However, at pH 8.0, the pH optimum for photophosphorylation, no stimulation of phosphorylation is observed. Nelson *et al.* (1972) concluded from their experiments with isolated coupling factor protein that HCO_3^- might cause a conformational change in this protein. This suggestion has been confirmed by Cohen and MacPeek (1980).

In this chapter, we will concentrate on the HCO_3^-* effects on photosynthetic electron transport. For a background on electron transport, the reader is referred to Vermaas and Govindjee (1981b) and Cramer and Crofts, Chapter 9, Vol. I (1982). No attempt is made to speculate on the "active species," i.e., the species (CO_2, HCO_3^- or CO_3^-) that is responsible for restoration of electron transport or about the molecular mechanism of the specific HCO_3^-* binding to the thylakoid membrane. For publications that cover these questions, see Govindjee and van Rensen (1978), Stemler (Chapter 15, this volume), Vermaas and Govindjee

Since it is unknown whether CO_2 or HCO_3^- is necessary for efficient electron transport, we will use the description HCO_3^- to denote the CO_2 or HCO_3^- that is specifically bound to the membrane and that exerts its effect on electron transport.

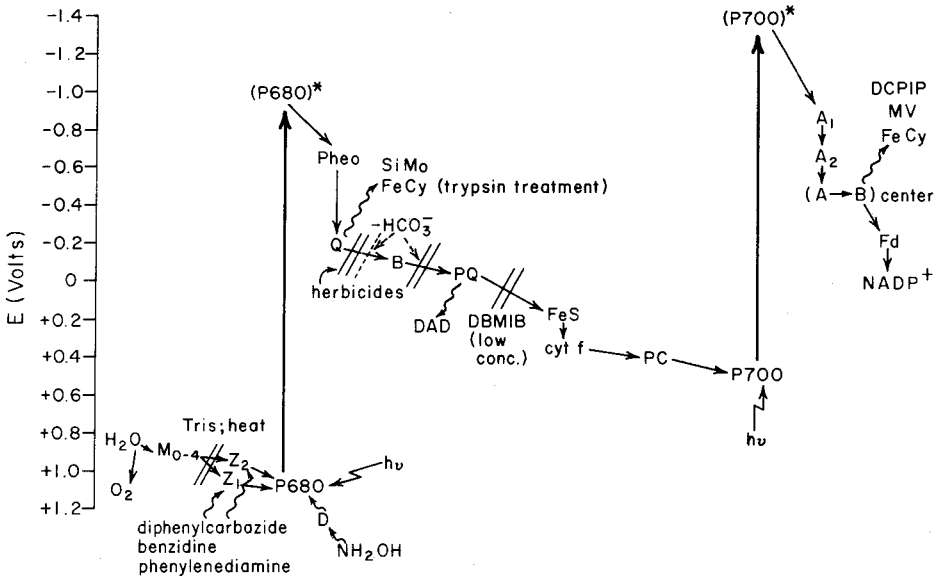

FIG. 1. Pathway of noncyclic electron flow from H_2O, the electron donor in photosynthesis, to $NADP^+$ (nicotinamide adenine dinucleotide phosphate), the "physiological" electron acceptor. E on the ordinate stands for midpoint redox potential. Light quanta ($h\nu$) are absorbed in two sets of antenna Chl molecules; the excitation energy is transferred to the reaction center Chl *a* molecules of PSII (P680) and of PSI (P700), which forms (P680)* and (P700)*, respectively. The latter two initiate electron transport. Inhibitors of photosynthetic electron transport are known to work at specific sites in the electron transport chain. Inhibition of electron transport by a certain treatment or molecule is indicated as $\not\rightarrow$, slowing down of electron transport as $\nrightarrow$, and electron donation or acceptance by an artificial donor or acceptor is drawn as a curved line. M_{0-4}, the charge accumulator of the oxygen-evolving system that can exist in several charged states; D, an unknown electron donor to P680; FeS, the Rieske iron–sulfur center; cyt *f*, cytochrome *f*; PC, plastocyanin; A_1, the primary PSI acceptor, is suggested to be a Chl molecule; A_2, an iron containing protein, which is considered equivalent to the so-called X; (A → B) center, iron–sulfur centers observed in EPR spectra; and Fd, ferredoxin. (Also see the List of Abbreviations.) (For further details, see Volume I, also edited by Govindjee, 1982.)

(1981a), Sarojini and Govindjee (1981), and Vermaas and van Rensen (1981).

II. A Site of HCO_3^-* Action on the Acceptor Side but Not on the Donor Side of Photosystem II

Warburg and Krippahl (1958, 1960) showed that HCO_3^-* is necessary for efficient electron transport in thylakoids or intact *Chlorella* cells,

using quinone as an artificial electron acceptor. After this discovery, several groups investigated this "bicarbonate effect" further (see, e.g., Abeles *et al.,* 1961; Stern and Vennesland, 1962; Good, 1963; West and Hill, 1967). These initial studies were limited due to variability in the CO_2-depleted material. Once a reproducible method for CO_2 depletion was developed (Stemler and Govindjee, 1973), a determination of the site of HCO_3^-* action was possible. Although at first, Stemler and Govindjee (1973) observed no bicarbonate effect (i.e., stimulation of electron transport by HCO_3^- addition) using DPC (diphenylcarbazide) and DCPIP (2,6-dichlorophenolindophenol) as artificial donor and acceptor, respectively, experiments performed later by Wydrzynski and Govindjee (1975) showed a significant bicarbonate effect on the DPC → DCPIP reaction, but the effect was lower than in the H_2O to DCPIP Hill reaction. These observations could indicate an action of HCO_3^-* both on the acceptor and on the donor side of PSII. However, care should be taken in interpreting these data because DPC might have effects on the electron transport chain other than electron donation to the donor Z of the PSII reaction center (P680), e.g., an increase in the efficiency of PSII (Harnischfeger, 1974). Fischer and Metzner (1981) showed that the methyl viologen-mediated Mehler reaction (monitoring noncyclic electron transfer through PSII and PSI) in thylakoids treated with hydroxylamine (blocking $P680^+$ reduction by the physiological donor Z and reducing $P680^+$ via an unknown donor D) is relatively insensitive to HCO_3^-*. However, Wydrzynski and Govindjee (1975) observed a large HCO_3^-* effect using NH_2OH as an electron donor. In order to resolve this uncertainty, the induction kinetics of the variable Chl *a* fluorescence after CO_2 depletion may be examined. The variable Chl *a* fluorescence monitors, among other things, the redox state of Q; Q is a quencher of Chl *a* fluorescence, whereas Q^- is not (see, e.g., a review by Lavorel and Etienne, 1977). Therefore, a rapid accumulation of Q^- due to an inhibition or slowing down of electron transport beyond Q is easily detected by fluorescence measurements. The CO_2 depletion causes a fast increase in the variable fluorescence yield (Stemler and Govindjee, 1974; Wydrzynski and Govindjee, 1975), similar to that observed in the presence of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (Wydrzynski and Govindjee, 1975); the latter blocks reoxidation of Q^- by $B^{(-)}$, the second quinone-type PSII acceptor. Blockage of the donor side by heat treatment (Katoh and San Pietro, 1967; Homann, 1968) causes a much lower yield of the variable fluorescence (Wydrzynski and Govindjee, 1975). Since fluorescence induction traces of CO_2-depleted chloroplasts resemble, on a short time scale, traces of normal chloroplasts in the presence of DCMU, but do *not* resemble traces in which the donor side is blocked, it may be concluded that the primary site of HCO_3^-* action is

not on the oxygen evolving system (Wydrzynski and Govindjee, 1975). Furthermore, after alkaline Tris-washing (which deactivates the oxygen-evolving system (Yamashita and Butler, 1968)) and CO_2 depletion of chloroplasts, the addition of artificial PSII electron donors (e.g., DPC and NH_2OH) is unable to change $-HCO_3^-$* fluorescence characteristics into $+HCO_3^-$* characteristics, which points to a bicarbonate effect between the site of donation by these external donors and PQ (Wydrzynski and Govindjee, 1975). If thoroughly CO_2-depleted chloroplasts are used, a major fast rise without any significant slow phase in the fluorescence transient is observed. This points to a (nearly) complete blockage of electron transport on the acceptor side of PSII (Vermaas and Govindjee, 1982). Since the area over the fluorescence induction curve in CO_2-depleted chloroplasts is about twice as large without DCMU than with it a major block must occur beyond B (Vermaas and Govindjee, 1982). If the CO_2 depletion is not complete, then a fast rise in the fluorescence induction curve followed by a slow rise to the maximum fluorescence level is observed (Stemler and Govindjee, 1974). (For a detailed explanation of the latter data, see Vermaas and Govindjee, 1981a.)

A. Electron Transport Rates in the Presence of Artificial Electron Acceptors

In the presence of SiMo (silicomolybdate; $SiMo_{12}O_{40}^{4-}$) and DCMU, no significant bicarbonate effect is observed in CO_2-depleted chloroplasts (Khanna *et al.*, 1977; van Rensen and Vermaas, 1981a). [SiMo is an artificial acceptor known to accept electrons from Q (Giaquinta and Dilley, 1975; Zilinskas and Govindjee, 1975).] It is difficult to imagine a significant CO_2-depletion effect on the donor side of PSII that would not inhibit electron flow from H_2O through P680 to SiMo. However, the addition of HCO_3^- causes a dramatic increase in the Hill reaction with oxidized DAD (2,3,5,6-tetramethylphenylenediamine) as acceptor when DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) is present to inhibit electron flow beyond the PQ pool (Khanna *et al.*, 1977; Sarojini *et al.*, 1981; see also Fig. 1). No measurable effect of HCO_3^-* is observed in a PSI reaction as monitored by electron flow from reduced DAD to MV (methyl viologen) in the presence of DCMU (Khanna *et al.*, 1977). These data indicate that the most important effect of HCO_3^-* is between Q and PQ and not on the donor side of PSII, since the reaction $H_2O \rightarrow$ SiMo appears to be almost insensitive to 10 m*M* bicarbonate. Further support against an effect of HCO_3^-* on the donor side of PSII is provided by data on trypsin-treated chloroplasts. Ferricyanide (FeCy) is known to be able to accept electrons directly from Q after trypsin treat-

ment (Renger, 1976; van Rensen and Kramer, 1979); after trypsin incubation, no bicarbonate effect on the FeCy Hill reaction is observed (Khanna *et al.*, 1981; van Rensen and Vermaas, 1981b), suggesting that HCO_3^-* does not influence the donor side of PSII.

B. Absorption Changes Due to Quinones

An independent although somewhat more complicated way to monitor electron transport is by spectrophotometry. Flash-induced absorption changes in the 320–334-nm region are ascribed to the oxidation or reduction of Q (Stiehl and Witt, 1968; Witt, 1973; van Gorkom, 1974; Renger, 1976; Siggel *et al.*, 1977), whereas changes in absorption at 265 nm are correlated with changes mainly in the B and PQ pool (Stiehl and Witt, 1969; Haehnel, 1976; Siggel *et al.*, 1977).

Using repetitive flashes (with 250 msec dark time between the flashes), a part of the Q^- decay, as measured by 334-nm absorbance changes, is slowed down significantly in CO_2-depleted chloroplasts [450 μsec (in control) → 6 msec ($-HCO_3^-$*)]. After HCO_3^- addition, this slow phase nearly disappears, and the kinetics become similar to that in the control. The slowing down of the Q^- decay in the absence of HCO_3^-* suggests a decrease in the rate of Q^- oxidation by B. Furthermore, the amplitude of the absorbance change at 334 nm is smaller before than after HCO_3^- addition (Siggel *et al.*, 1977). This was interpreted as a (reversible) inactivation of P680 (Siggel *et al.*, 1977). However, no bicarbonate effect on steady state electron transport from H_2O to Q has been detected (Khanna *et al.*, 1977; van Rensen and Vermaas, 1981a); so, the explanation of a reversible P680 inactivation by CO_2 depletion is not supported by other data. A more reasonable explanation for this phenomenon is that part of Q^- is reoxidized very slowly in the absence of HCO_3^-* (~ 1 sec) (Jursinic and Stemler, 1981). Therefore, part of Q^- formed after one flash is not yet reoxidized when the next flash arrives at the sample; this results in a lower [Q] just before a flash and in a lower amplitude of the absorbance change at 334 nm in the absence of HCO_3^-*. It is not yet known if this very slow component of Q^- reoxidation is due to electron transfer to $B^{(-)}$ or to another electron acceptor. This means that certain PSII reaction centers are not completely "turned off" by CO_2 depletion, but rather have had no time to recover between flashes. Thus, absence of HCO_3^-* does not decrease the number of active reaction centers (P680).

When absorption changes at 265 nm that are induced by 85 msec flashes (spaced 5 sec apart) are compared in CO_2-depleted chloroplasts with and without HCO_3^-* addition, it is observed that CO_2 depletion

reduces the amplitude of the 265 nm signal and slows down its decay. The kinetics of formation of the signal are determined by the opening time of the shutter (~ 10 msec) and are not significantly influenced by HCO_3^-*. To explain these data, it was suggested that in the absence of HCO_3^- the reduction of the PQ pool is slow compared to its oxidation, causing a low steady state [PQH_2] during illumination and, therefore, a small absorbance change. The observed absorbance change was interpreted to be due mainly to the formation of B^{2-}.† Then, the slow decay has to be ascribed to a slow electron transport from B^{2-} to PQ ($t_{1/2}$ ~ 100 msec), followed by a relatively fast reoxidation of PQH_2 ($t_{1/2}$ ~ 25 msec) (Siggel *et al.*, 1977). Further experiments are needed to confirm these interpretations.

C. *Chlorophyll a Fluorescence Yields after Light Flashes*

Experiments pointing to a significant slowing down of the oxidation of B^{2-} by PQ in CO_2-depleted chloroplasts were presented by Govindjee *et al.* (1976). When CO_2-depleted chloroplasts are subjected to one or two saturating flashes, spaced approximately 30 msec apart, the Chl *a* fluorescence yield 160 msec after the flash(es) are as low as in CO_2-depleted chloroplasts after HCO_3^- addition (Fig. 2). This observation indicates a reasonably fast (< 30 msec) reoxidation of Q^- after the first two flashes in CO_2-depleted chloroplasts. However, when three or more flashes are applied the fluorescence yield (and, thus, the Q^- concentration) 160 msec after the last flash is much higher in these chloroplasts than in CO_2-depleted chloroplasts to which HCO_3^- is added (Fig. 2). These data show a relatively efficient electron transport from Q^- to $B^{(-)}$ of only two electrons in CO_2-depleted chloroplasts. This is the number of electrons that can be accepted by B after dark adaptation when there is no appreciable electron transport from B to PQ. Although the dark time between the flashes in these experiments was somewhat short (not all Q^- after the first flashes is oxidized before the next flash reaches the sample; see Jursinic and Stemler, 1981), these data strongly imply the existence of the main site of HCO_3^-* action on the oxidation of B^{2-} instead of on the oxidation of Q^-. If the oxidation of Q^- were to be slowed down by CO_2 depletion into the 100 msec range, the fluorescence 160 msec after the first two flashes would be as high as after the other flashes. This was not observed (Fig. 2). Therefore, these data point to a lack of reoxidation of B^{2-} and, thus, to a severe slowing down of electron transport from B to PQ. However, quantitative analyses require further experiments.

†See footnote on p. 542.

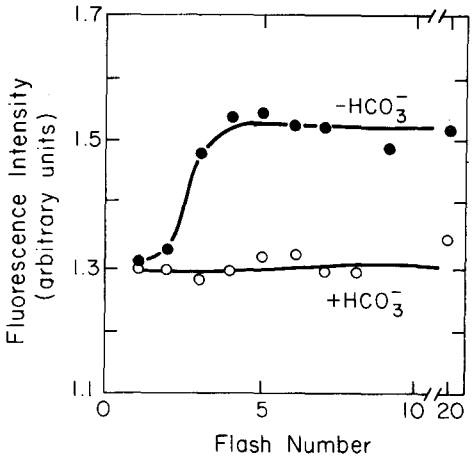

FIG. 2. Intensity of Chl *a* fluorescence 160 msec after the last of a series of 3 μsec saturating flashes, spaced approximately 30 msec apart, as a function of the number of flashes in CO_2-depleted chloroplasts with (○) or without (●) 20 m*M* $NaHCO_3$; 20 μg Chl ml^{-1}. The reaction mixture contained 50 m*M* Na phosphate, 100 m*M* NaCl, 100 m*M* Na formate (pH, 6.8). (From Govindjee *et al.*, 1976.)

The preceding conclusion is confirmed by experiments in which DCMU is added to a sample after a certain number of flashes. DCMU addition is known to shift the $Q^-B \rightleftharpoons QB^-$ equilibrium to the left (Velthuys and Amesz, 1974) and is a useful probe to monitor the redox state of B by means of fluorescence, which is sensitive to the redox state of Q. In CO_2-depleted chloroplasts to which HCO_3^- is added, a damped oscillation of fluorescence as a function of flash number (with a period of 2) is observed when DCMU is added before fluorescence is measured (Fig. 3). This observation is expected if electron transport between B^{2-} and PQ is relatively fast. B is mainly in the oxidized form after dark adaptation, and DCMU addition does not cause a large back reaction (i.e., oxidation of B^- or, perhaps, B^{2-} by Q) because $[B^-]$ is low and $[B^{2-}]$ negligible. After one flash, B is mainly in the B^- form just before DCMU is added (B^- is relatively stable), resulting in a high fluorescence yield. After two flashes, B is mainly fully oxidized (the B^{2-} oxidation by PQ is much faster than the DCMU addition). The third and fourth flashes give results similar to the first and second flashes, respectively. However, in CO_2-depleted chloroplasts such an oscillation with a period of 2 is not observed (Fig. 3); after dark adaptation the fluorescence yield is low, whereas after one flash a high fluorescence intensity is observed. The fluorescence yield after DCMU addition remains high after the subsequent flashes, pointing to a high relative concentration of B^- and/or B^{2-} in these samples after one or more flashes. A reasonable explanation for this observation is to assume that most of the reduced B formed by a flash is still present when DCMU is added. This indicates a prolonged lifetime of reduced B in CO_2-depleted chloroplasts (Govindjee *et al.*, 1976).

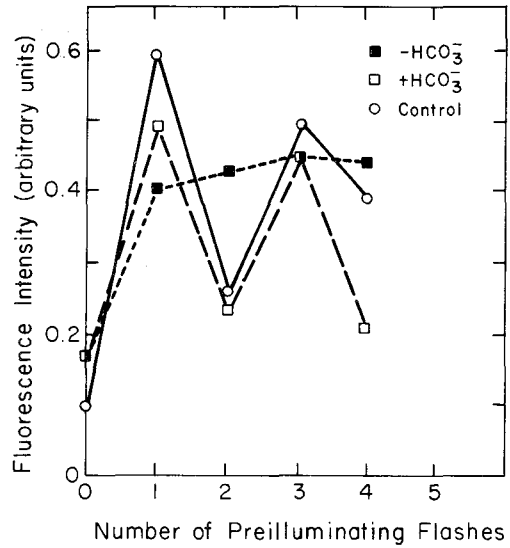

FIG. 3. DCMU-induced increase in Chl *a* fluorescence as a function of the number of preilluminating flashes. 20 μg Chl ml^{-1}; ○, non-depleted (control) chloroplasts; ■, CO_2-depleted chloroplasts; □, CO_2-depleted chloroplasts + 20 m*M* $NaHCO_3$. Reaction medium as described in the legend of Fig. 2. (From Govindjee *et al.*, 1976.)

D. Light-Induced Proton Uptake and Release by the Thylakoid Membrane

Proton uptake and release by thylakoid membranes can be monitored spectrophotometrically using indicator dyes and appropriate buffers in an accurate and elegant manner (Junge and Ausländer, 1973; Junge *et al.*, 1979). When flash-induced pH changes in the internal space of the thylakoid are measured in CO_2-depleted chloroplasts, the amplitude of the rapid pH change due to proton production by the oxygen-evolving system is decreased compared to control chloroplasts, while a slow component ($t_{1/2} \sim 100$ msec), present in control chloroplasts, disappears (Khanna *et al.*, 1980). This slow component is related to the oxidation of PQH_2, which causes proton movement into the inside (Ausländer and Junge, 1975). The absence of this component indicates that PQH_2 is formed only very slowly in CO_2-depleted chloroplasts (Khanna *et al.*, 1980). The decrease in amplitude of the rapid phase (due to H^+ release into the intrathylakoid space) was interpreted by Khanna *et al.* (1980) as indicating an inactivation of PSII. This is probably due to an irreversible inactivation by the depletion procedure, and not to a reversible inactivation by a HCO_3^-* effect on the Q^- oxidation as the dark time between the flashes (10 sec) was long enough to obtain full recovery of Q. Khanna *et al.* (1980) could not determine the reversibility of these effects because readdition of HCO_3^- caused an increase in the buffering capacity of the

system, resulting in a dramatic decrease of the pH changes. Further experiments are needed to test the reversibility of these effects. Our tentative interpretation is that CO_2 depletion affects the PQH_2 formation much more than the O_2 evolution. (The changes in the PQH_2 formation rate are expected to be reversible by HCO_3^- addition, whereas the change in O_2 evolution is probably an irreversible "artifact" of the depletion procedure.)

E. Reduction of the Electron Donor Z and of the Photosystem II Reaction Center

To investigate if HCO_3^-* causes changes in $P680^+$ and Z^+ reduction kinetics, which would be expected if HCO_3^-* had any effect on the donor side of PSII, fluorescence measurements in the μsec range were performed, and the decay kinetics of the EPR signal II_{vf} were recorded (Jursinic *et al.*, 1976). If repetitive flashes are used and, thus, the $P680^+$ reduction by Z slowed down into the (sub) μsec range (e.g., Sonneveld *et al.*, 1979), the rise in Chl *a* fluorescence yield in the μsec range is indicative, among other things, of the rate of electron flow from Z to $P680^+$ because $P680^+$ is known to be a quencher of fluorescence (Okayama and Butler, 1972; Butler *et al.*, 1973). Therefore, $P680^+$ reduction results in an increase in fluorescence yield. These rise kinetics are not influenced by HCO_3^-* (Jursinic *et al.*, 1976), indicating that the $P680^+$ reduction by Z is insensitive to HCO_3^-*. The EPR signal II_{vf} kinetics, monitoring the reduction of Z^+ by the oxygen-evolving system (Blankenship *et al.*, 1975), which were measured in an experiment using repetitive flashes, are also unchanged by CO_2 depletion (Jursinic *et al.*, 1976). However, the amplitude of the signal is decreased by 40% in the absence of HCO_3^-*. One explanation for this decrease is that the dark time between the flashes (1 sec) was not long enough to reoxidize Q^- completely (the Q^- reoxidation in CO_2-depleted chloroplasts has a very slow component as mentioned previously). The P680* (i.e., P680 in the excited state) cannot transfer an electron to Q^-, and there is no stable charge separation. For this reason, a non-negligible portion of Z is not oxidized after a flash, and the EPR II_{vf} signal is expected to be lower in CO_2-depleted chloroplasts.

In our opinion, the absence of a significant change in the EPR II_{vf} decay kinetics in CO_2-depleted chloroplasts, monitoring the reduction of Z^+ by the oxygen-evolving system, rules out under these experimental conditions any significant role of HCO_3^-* on the oxygen-evolving system, whereas the unchanged μsec fluorescence kinetics indicate that the electron donation to $P680^+$ is not influenced by HCO_3^-*.

*F. Relationship between Herbicides and HCO_3^-**

Experiments in which $H^{14}CO_3^-$ is added to CO_2-depleted chloroplasts and the $H^{14}CO_3^-$* binding monitored as a function of the $H^{14}CO_3^-$* concentration suggest that there is only one specific HCO_3^-* binding site per PSII reaction center (Stemler, 1977). Furthermore, it has been shown that the DCMU and HCO_3^-* binding sites are close together because $H^{14}CO_3^-$* removal by SiMo washing is greatly decreased by prior addition of DCMU (Stemler, 1977). A close spatial relationship between the HCO_3^-* and herbicide binding sites has now been described (Khanna *et al.*, 1981; van Rensen and Vermaas, 1981b; Vermaas *et al.*, 1982). Since herbicides are known to bind to a surface-exposed protein, very close to Q and B, and to be accessible to degradation by trypsin (Renger, 1976; Trebst, 1979; Arntzen *et al.*, 1981), it is reasonable to assume that HCO_3^-* binds to the same or to an adjacent protein molecule. This explains why a HCO_3^-* interaction with the oxygen-evolving system or even with the donor side of PSII, although possible, is unlikely.

III. Postulated Effects of HCO_3^-* on the Donor Side of Photosystem II

There are some HCO_3^-* effects that are easily explained by assuming HCO_3^-* action on the water splitting system directly (see Stemler, Chapter 15, this volume). (For a recent review on water oxidation, S-states, and O_2 evolution, see Wydrzynski, Chapter 10, Vol. I, 1982.) However, here we argue that these data do not necessarily indicate a HCO_3^-* influence even on the donor side of PSII.

A. Effects on the S-States

When oxygen evolution in CO_2-depleted chloroplasts is measured as a function of flash number, the damping of the oscillation with a period of 4 (Kok *et al.*, 1970) is faster without HCO_3^- addition than with it (Stemler *et al.*, 1974). This indicates that the miss parameter α, the probability of not undergoing a net change in the charge accumulator M after a flash, is higher in the absence of HCO_3^-*. This might be due to a HCO_3^-* effect on the donor side, but is equally well explained by an effect on the acceptor side. If Q^- is not fully reoxidized in the dark time between the flashes (1 sec), which is probably the case (Jursinic and Stemler, 1981), then a certain number of PSII centers (those of the type

P680.Q^-) cannot transfer an electron from P680* to the first quinone acceptor as it is in Q^- form and, therefore, no change is observed in those centers. Assuming that the slowly oxidizing Q^- does not belong to a special type of PSII center, it is obvious that the high α value in CO_2-depleted chloroplasts can readily be explained by a HCO_3^-* effect on the Q^- reoxidation.

Furthermore, it is known that the time necessary to allow another turnover of the charge accumulator M (often denoted as the $S'_n \rightarrow S_{n+1}$ reaction rate) is extended by a factor of 10 or more by CO_2 depletion (600 μsec → 10 msec) (Stemler *et al.*, 1974). This time necessary for "relaxation" (i.e., the $S'_n \rightarrow S_{n+1}$ reaction) is the time that it takes to open the PSII trap, i.e., to oxidize Q^- or to reduce $P680^+$, whichever is slower. Since the Q^- oxidation is slowed down by CO_2 depletion to a value of at least 4–10 msec (Jursinic *et al.*, 1976; Siggel *et al.*, 1977), it is probable that the slowing down of the $S'_n \rightarrow S_{n+1}$ reaction rate is caused by an effect of HCO_3^-* on the reoxidation of Q^-. Under these circumstances, the very slow component of Q^- reoxidation does not seem to show up in the rate of the $S'_n \rightarrow S_{n+1}$ reaction.

The kinetics of oxygen evolution ($S_4 \rightarrow S_0$) as measured by an unmodulated Joliot electrode are not affected by HCO_3^-* at pH 6.8 (Stemler *et al.*, 1974). However, at pH 5.3, small differences in the kinetics of O_2 evolution were detected as stated in Chapter 15 ($t_{1/2} = 4.93 \pm 0.18$ msec, $+HCO_3^-$*; $t_{1/2} = 5.52 \pm 0.27$ msec, $-HCO_3^-$*) (Stemler, 1981); these differences are very small as compared to other HCO_3^-* effects, and they could not be confirmed by the authors (Fig. 4).

Sodium formate ($NaHCO_2$), which seems to compete with HCO_3^-* (N. Good, unpublished; Khanna *et al.*, 1977; Vermaas and van Rensen, 1981), was shown to lengthen the relaxation time of $S'_2 \rightarrow S_3$ and $S'_3 \rightarrow S_0$ at pH 8.2, without affecting $S'_0 \rightarrow S_1$, $S'_1 \rightarrow S_2$, α and the "double hit" parameter β (Stemler, 1980a). Stemler proposed that those S-state transitions that show an extended relaxation time in the presence of formate must result in momentary release and rebinding of CO_2 (see Stemler, Chapter 15, this volume). If formate under these conditions would be able to replace HCO_3^-*, then a higher α value should be obtained in the presence of formate than in the absence of formate, whereas $S'_0 \rightarrow S_1$ and $S'_1 \rightarrow S_2$ also should be slowed down because of the slowing down of Q^- decay in the absence of HCO_3^-*. This was not observed. Therefore, we suggest that under these conditions (pH 8.2) formate may not be able to remove HCO_3^-* from its binding site. The differences that were observed in the $S'_2 \rightarrow S_3$ and $S'_3 \rightarrow S_0$ reactions in the presence and absence of formate could also be explained by a formate effect, other than HCO_3^-* removal, which affects S_2 and S_3 specifically. Bouges-

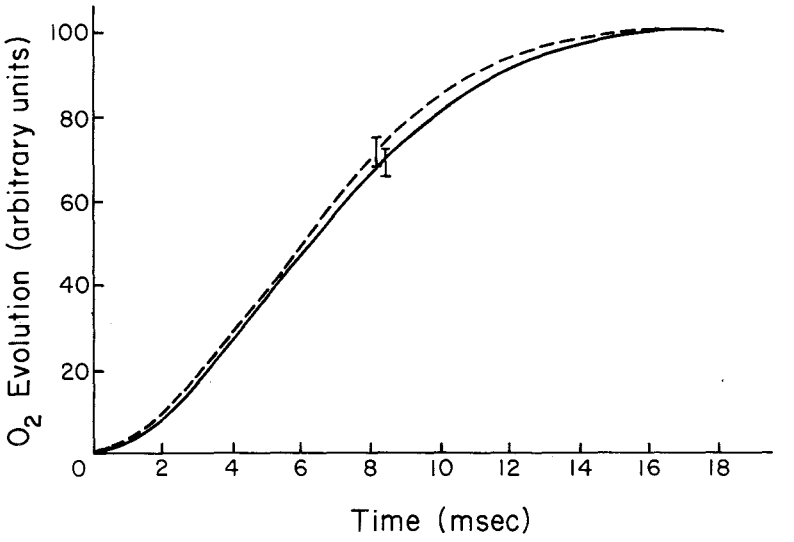

FIG. 4. Oxygen evolution, after the third flash, as a function of time in the presence (——) and the absence (- - -) of 10 m*M* $NaHCO_3$. Measurements were made with an unmodulated Joliot electrode. CO_2-free medium consisted of 50 m*M* Na phosphate, 100 m*M* $NaHCO_2$, 100 m*M* NaCl, 5 m*M* $MgCl_2$, pH, 5.3, and CO_2-sufficient medium was 50 m*M* Na phosphate, 100 m*M* $NaHCO_2$, 10 m*M* $NaHCO_3$, 90 m*M* NaCl, 5 m*M* $MgCl_2$, pH, 5.3. Xenon flashes, $\leq$ 10 μsec; number of experiments, 5. Note the absence of decrease in $t_{1/2}$ of the signal rise upon addition of CO_2. (Data of W. F. J. Vermaas and Govindjee, unpublished observations, 1980–1981.)

Bocquet (1980) suggested that two different electron donors, Z_1 and Z_2, donate electrons to P680 in parallel. Both of them are related to transitions in two S-states. Z_1 is connected to S_0 and S_1, and Z_2 is connected to S_2 and S_3. Thus, formate may slow down the Z_2 reduction but not the Z_1 reduction.

It is difficult to understand why at pH 8.2 formate does not affect $S'_0 \rightarrow S_1$ and $S'_1 \rightarrow S_2$ transitions, whereas at low pH those transitions are affected, but differentially (see Chapter 15). In Stemler's hypothetical scheme of CO_2 involvement in oxygen evolution, $S'_0 \rightarrow S_1$ and $S'_1 \rightarrow S_2$ would not be influenced by HCO_3^-* (Stemler, 1980a), and, therefore, this scheme does not explain his $S'_0 \rightarrow S_1$ and $S'_1 \rightarrow S_2$ data at pH 5.3 either. A possible explanation is that the Q^- reoxidation is S-state dependent. It is known that the rate of Q^- reoxidation by a back reaction is dependent on the S-state (Joliot *et al.*, 1971; Lavergne and Etienne, 1982), but more experiments are needed to check if such a statement can explain the observed difference under these low pH conditions.

B. $H^{14}CO_3^-$* Binding Studies

Stemler (1980b) showed that the rate of $H^{14}CO_3^-$* binding to CO_2-depleted chloroplasts is pH independent in the first 2 mins. However, when the thylakoids are equilibrated for 5 min followed by $H^{14}CO_3^-$* addition, then the binding is faster at low pH than at high pH (pH 6.0 versus 7.8). These results were interpreted by Stemler (1980b) to suggest that the internal pH (which was assumed to be equal to the external pH after 5 min equilibration but not in the first 2 mins) rather than the external pH governs HCO_3^-* binding. Since the oxygen-evolving site is located on the inner side of the thylakoid membrane, an interaction of HCO_3^-* with the oxygen-evolving system was suggested (see Stemler, Chapter 15, this volume). However, CO_2-depleted chloroplasts are uncoupled by the depletion procedure (Khanna *et al.*, 1977) and, thus, a fast pH equilibration (in the second range) between the inside and the outside of the thylakoid vesicle may be obtained. Furthermore, even in nondepleted (control) chloroplasts, the pH equilibration over the thylakoid membrane is rather rapid (rate constant of proton leakage: 0.73 sec^{-1}) (Khanna *et al.*, 1980). This means that if the internal pH is important, differences in $H^{14}CO_3^-$* binding are expected to be lost within a couple of seconds. This was not observed. Further theoretical and experimental analyses are required, and, in our opinion, these data cannot yet be used to support HCO_3^-* binding to the inside of the thylakoid membrane.

IV. Conclusions

Although it has been assumed that HCO_3^-* may act on the donor side of PSII, perhaps even as a direct source of O_2 produced in the oxygen-evolving system (Warburg and Krippahl, 1958, 1960; Stemler, 1980a), there is an overwhelming amount of experimental observations now that support a major HCO_3^-* function between Q and PQ on the acceptor side of PSII (for earlier reviews, see Govindjee and van Rensen, 1978; Jordan and Govindjee, 1980; Vermaas and Govindjee, 1981a).

We believe that there are, as yet, no clear experimental results that point to any HCO_3^-* function on the donor side of PSII (Section III). In our opinion, the hypothetical scheme for a HCO_3^-* function in oxygen evolution as presented by Stemler (Chapter 15, this volume) is not based on convincing experimental data. We prefer a scheme in which HCO_3^-* binds to a site on or close to the herbicide binding protein, causing a conformational change in that protein that results in an allowance of

efficient electron transfer between Q and PQ (e.g., see Khanna *et al.*, 1981; Vermaas and van Rensen, 1981). Clearly, more experiments are needed to understand the molecular mode of action and the function of the bicarbonate effect in intact systems.

Acknowledgments

The authors thank John Whitmarsh for critical reading of the manuscript. We thank the National Science Foundation Grant PCM 24532 for financial support during the preparation of this chapter.

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