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Unique Role(s) of Carbon Dioxide and Bicarbonate in the Photosynthetic Electron Transport System†

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Photosynthesis utilizes CO_2, H_2O and light energy to produce O_2 and carbohydrates in chloroplasts. CO_2 is fixed into carbohydrates through the well-known Calvin-Benson-Bassham cycle or, in some plants, through the Hatch-Slack pathway. The present review deals with an additional and a unique role of CO_2 or bicarbonate (HCO_3^-) which is different from this well-established role of CO_2-acceleration of the photosynthetic electron transport from H_2O to nicotinamide adenine dinucleotide phosphate ($NADP^+$). CO_2-depletion of thylakoid membranes results in an inhibition of electron transport, while the readdition of bicarbonate restores this activity. Most of the experimental evidence, reviewed here, suggests that this so-called "bicarbonate effect" is located on the reducing (quinones Q, B and plastoquinone), and not on the oxidizing (water) side of photosystem II. The most favourable hypothesis is that CO_2 diffuses to the binding site (suggested to be a protein associated with the quinone B), while HCO_3^- binds to this site which may be in a "pocket" in the membrane. This may cause a conformational or chemical change in the protein allowing efficient electron flow from one quinone to the other (e.g. from Q^- to B, and from B^{2-} to plastoquinone).

Key Words: **Bicarbonate effect, Carbon dioxide, Electron transport, Photosynthesis, Thylakoid membranes**

I. Introduction

As early as the 18th century, it was observed that "fixed air" (CO_2) is necessary for oxygen production by green plants under the influence of light (Priestley 1776, Ingenhousz 1779, Senebier 1782). This CO_2 utilization is located in the "dark" reactions of photosynthesis: CO_2 acts as a substrate for ribulose-1, 5-bisphosphate

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carboxylase, which catalyzes the carboxylation of ribulose 1, 5 bisphosphate; the latter is the first step in the chain of reactions resulting in the conversion of CO_2 and NADPH (reduced nicotinamide adenine dinucleotide phosphate) into carbohydrates. Thus, the absence of CO_2 results in an inhibition of the use of NADPH formed at the end of the electron transport chain (figure 1). In this case, no net O_2 evolution occurs because the $NADP^+$ concentration is very low (there is a block in the utilization of NADPH), and Fd (ferredoxin) in its reduced form probably transfers electrons to O_2 instead of to $NADP^+$ (pseudocyclic photophosphorylation; Simonis & Urbach 1973, Egneus et al. 1975). CO_2 acts not only as a substrate, as mentioned above, but is also needed as an activator of ribulose-1, 5-bisphosphate carboxylase; CO_2 binding to an ϵ-amino group of a lysyl residue of the enzyme (Lorimer et al. 1976), leading to carbamate formation ($R\text{-}NH\text{-}COO^-$, in which R is the enzyme) (Lorimer & Miziorko 1980), is necessary to bring the enzyme into an active conformation. Therefore, CO_2 acts both as an activator and a substrate for ribulose-1, 5-bisphosphate carboxylase.

There are other less well-known, but important effects of CO_2 or bicarbonate (HCO_3^-) on the light-driven processes in photosynthesis—on photophosphorylation and on electron flow between the two photosystems (figure 1). The first effect is on the ATP synthesis driven by a proton gradient across the thylakoid membrane. It is generally accepted now that photosynthetic electron transport gives rise to a proton gradient across the thylakoid membrane (directed inwardly) (Neumann & Jagendorf 1964), and this proton gradient, along with the membrane potential gradient, is used for ATP synthesis (Mitchell 1961, 1966) by an enzyme complex, called coupling factor, in the thylakoid membrane (Avron 1963, Jagendorf 1975). Punnett and Iyer (1964) showed that photophosphorylation is enhanced by HCO_3^- addition to thylakoids at pH=7.0–7.5. These observations were extended by Nelson et al. (1972) to the isolated coupling factor protein and it was suggested that HCO_3^- might cause a conformational change in this protein. Recently, this suggestion has been confirmed (Cohen & MacPeek 1980); the stimulatory effect of HCO_3^- on ATP synthesis might be related to its ability to alter directly the conformation of the chloroplast coupling factor under conditions where the enzyme shows minimal activity due to suboptimal pH. At pH=8.0, the pH optimum for photophosphorylation, no stimulation of phosphorylation by HCO_3^- is observed.

The other important effect of CO_2 or bicarbonate is on photosynthetic electron transport directly — absence of CO_2 or bicarbonate inhibits electron transport, presumably between the first quinone-type photosystem II (PS II) acceptor, Q, and the plastoquinone pool, i.e. at the connection between Photosystems II and I (figure 1). This review will deal with this CO_2/bicarbonate action, which is referred to as "the bicarbonate effect". Neither the mechanism of this CO_2/HCO_3^- action, nor the nature of the binding species (CO_2 or HCO_3^-) is known. For this reason, we will use the symbol "HCO_3^{-*}" to indicate the species which binds to the specific binding site.

Warburg and Krippahl (1958) were the first to show that the Hill reaction with quinone as an artificial electron acceptor was dependent on the presence of HCO_3^{-*}, not only in kohlrabi thylakoids but also in intact *Chlorella* cells. These results were interpreted by Warburg (1964) as "proof" that CO_2 and not H_2O is the source of oxygen evolved in photosynthesis. This CO_2 requirement of the Hill reaction was confirmed by other groups in the early 1960's (Abeles et al. 1961, Stern & Vennesland

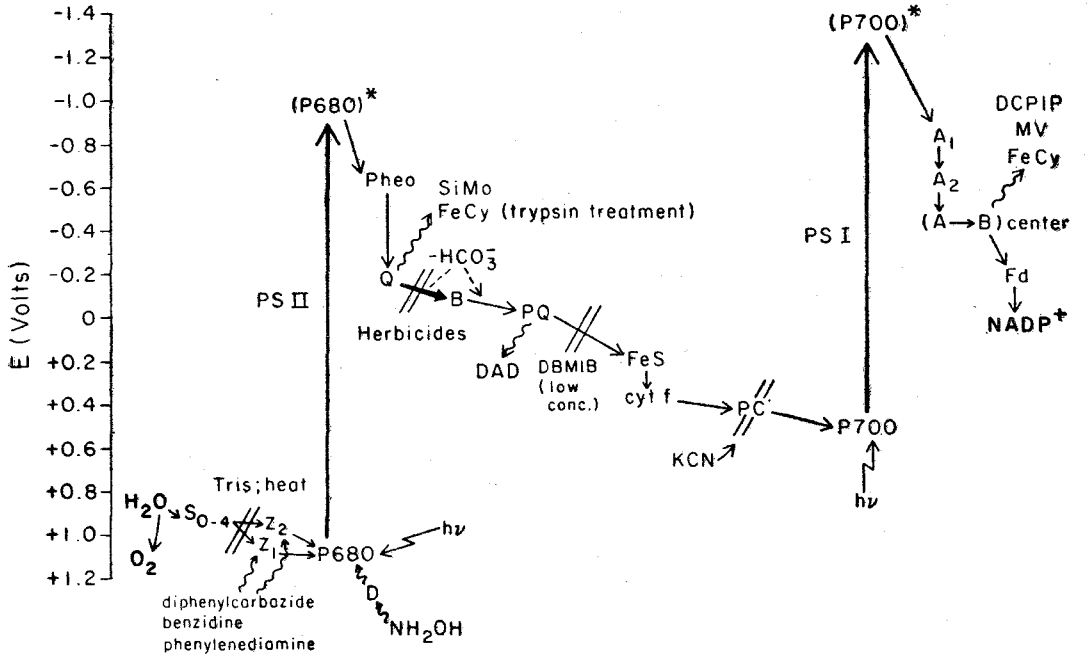

Figure 1 Pathway of noncyclic electron flow from H_2O, the electron donor of photosynthesis, to nicotinamide adenine dinucleotide phosphate ($NADP^+$), the "physiological" electron acceptor. E on the ordinate stands for midpoint redox potential. Light quanta ($h\nu$) are absorbed in two sets of antenna chlorophyll molecules, the excitation energy is transferred to the reaction center chlorophyll *a* molecules of photosystem II (P680) and of photosystem I (P700) forming (P680)* and (P700)*, and the latter two initiate electron transport. Inhibitors of photosynthetic electron transport and artificial electron donors or acceptors 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 —//→ , slowing down of electron transport as —/·→ , and electron donation or acceptance by an artificial donor or acceptor is drawn as a squiggled line. S_{0-4} refers to the charge-accumulator of the oxygen-evolving system that can exist in several charged states; Z_2 and Z_1 are the electron donors to P680; D is an unknown electron donor to P680; FeS represents the Rieske iron-sulfur center; Cyt f stands for cytochrome f; PC is plastocyanin; A_1 is suggested to be a chlorophyll molecule; A_2 is an iron-containing protein which has one of its absorbance bands at 430 nm and is considered equivalent to the so-called X; (A→B) center refers to iron-sulfur centers observed in electron spin resonance spectra and related to the so-called P430; and Fd stands for ferredoxin. The other abbreviations are explained in the text.

1962, Izawa 1962; also see Heise and Gaffron 1963). Good (1963) showed that anions that structurally resembled HCO_3^-, like acetate ($CH_3CO_2^-$) and formate (HCO_2^-), added in mM concentrations, were able to increase the dependence on HCO_3^{-}* significantly. The restoration of Hill reaction activity by HCO_3^- addition to CO_2-depleted chloroplasts was shown to be very specific for HCO_3^{-}*; phosphate, pyrophosphate, arsenate, nitrate, malonate, trimethylacetate, p-hydroxylbenzoate, glycine and tricine were not able to relieve the inhibition caused by CO_2 removal. Glycolate and maleate were the only ions that restored, although to a minor extent, the Hill reaction activity.

Beginning in the early 1970's, this bicarbonate effect on photosynthetic electron transport was investigated more thoroughly. Most of the experimental data showed that the main site of HCO_3^{-}* was not on the oxygen-evolving side of PS II as thought initially (Warburg & Krippahl 1958), but between Q and the PQ (plastoquinone) pool. However, some recent data are interpreted as suggesting a direct involvement of HCO_3^{-}* in oxygen evolution (Stemler 1980 a,b,c).

This review will stress recent progress in the "bicarbonate field" and deal with the following questions: (a) Where is the site of action of HCO_3^{-}*? (b) What is the "active species" which binds to a specific site and restores electron flow? And, what could be the molecular binding mechanism? For older reviews concerning this subject, see Govindjee and van Rensen (1978) and Jordan and Govindjee (1980).

II. The Site(s) of HCO_3^{-}* Action

Stemler and Govindjee (1973) suggested that the bicarbonate effect might be located on the oxidizing (i.e. "water") side of PS II. This suggestion was based on the HCO_3^{-}*-independence of the electron flow from diphenylcarbazide (DPC), an artificial PS II donor, to dichlorophenolindophenol (DCPIP) in heat-treated thylakoids. Heat treatment inactivates the oxygen-evolving system as shown by Katoh and San Pietro (1967) and Homann (1968). However, Wydrzynski and Govindjee (1975) showed a significant bicarbonate effect, although not as large as from H_2O to DCPIP, on the DPC→DCPIP reaction after inactivating the oxygen-evolving system by alkaline Tris treatment. It is not yet known why the DPC→DCPIP reaction did not show the full bicarbonate effect: was it because HCO_3^{-}* had an effect on the oxygen-evolving system in addition to that on electron transport on the reducing side of PS II, or because DPC also has effects other than electron donation to Z^+? (Z is the electron donor to the reaction center chlorophyll *a* P680; figure 1.) One side-effect of DPC might be an increase in the efficiency of PS II (Harnischfeger 1974). We favour the second possibility because the benzidine → DCPIP reaction is as sensitive to HCO_3^- as the H_2O→DCPIP reaction (Vermaas & van Rensen, unpublished) (benzidine is also a PS II donor). This indicates that there is no major site of HCO_3^{-}* action related to the oxygen-evolving system, and, indeed, nearly all the data obtained thus far can be explained by assuming that the important site of HCO_3^{-}* action is on the reducing side of PS II between Q and PQ.

II.A. *BICARBONATE EFFECTS ON THE REDUCING SIDE OF PS II*

II.A.1. *Chlorophyll a fluorescence measurements*

Chlorophyll (chl) *a* fluorescence is an excellent indicator of photosystem II reactions of photosynthesis (for details, see reviews by Papageorgiou (1975); Lavorel and Etienne (1977); Govindjee and Jursinic (1979)). When dark-adapted samples are illuminated, chl *a*

fluorescence intensity changes with time: an instantaneous increase to a level (0 level) is followed by a slower increase to a maximum level (P level).

To explain chl *a* fluorescence transients in the ms–s range, it is generally assumed that the yield of variable fluorescence is a function of the redox state of Q (Duysens & Sweers 1963): Q in its oxidized form is a quencher of PS II fluorescence, while Q in its reduced form (Q^-) is not. Chlorophyll fluorescence yield at a certain time is proportional to the concentration of Q^-([Q^-]) which in turn is dependent upon the rate of its formation by PS II reactions and the rate of its disappearance by reoxidation; the fluorescence induction transients obtained by illumination of dark-adapted samples, thus, monitor the redox state of Q with time.

The fluorescence induction curves of CO_2-depleted chloroplasts before and after HCO_3^- addition are shown in figure 2, [Q^-] in the absence of HCO_3^-* is higher at short time scales (up to ~ 700 ms), but lower at longer time scales in comparison with [Q^-] in the presence of HCO_3^-* (figure 2, insert). This was explained by a HCO_3^-* action prior to Q (Stemler & Govindjee 1974). However, a slowing down of electron transport between Q and PQ in the absence of HCO_3^-* explains these data more readily (also see figure 1). A slower electron flow from Q^- to PQ allows an initial increase in the concentration of Q^- to a certain level, but the same leads to a slower filling of the PQ pool and, thus, it takes a longer time to reach the maximum concentration of Q^-. We now explain this more fully. Reoxidation of Q^- by a two-electron acceptor B, which is the second quinone type PS II acceptor, and reoxidation of B^{2-} by PQ are much slower in the $-HCO_3^-$ preparations compared to the $+HCO_3^-$* samples (Jursinic et al. 1976, Govindjee et al. 1976, Siggel et al. 1977) causing a higher [Q^-] in "$-HCO_3^-$ chloroplasts" compared to "$+HCO_3^-$ chloroplasts" shortly (<~700 ms) after the onset of illumination. This is followed by a slower rise in $-HCO_3^-$* samples in comparison to $+HCO_3^-$* samples. Since electron transport from Q to PQ is slowed down in the absence of HCO_3^-*, it takes longer to fill

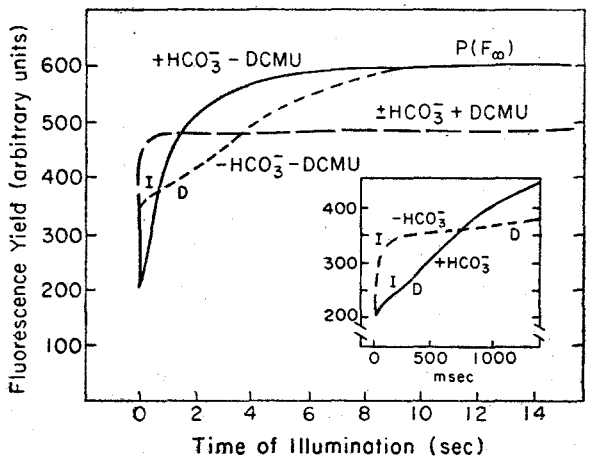

Figure 2 Fluorescence yield of chlorophyll *a* at 685 nm as a function of time of illumination in the presence and the absence of 10 mM $NaHCO_3$. Maize chloroplasts, depleted of HCO_3^-*, were suspended in 250 mM NaCl, 40 mM Na acetate, 50 mM phosphate buffer *p*H=6.8, $\pm 10^{-5}$ M DCMU; 10μg chl. ml^{-1}. Symbols I, D and P refer to certain points on the fluorescence transient (for definition, see Papageorgiou 1975) *(From Stemler and Govindjee 1974.)*

the PQ pool when no HCO_3^-* is present. Therefore, Q^- can be oxidized by B for a longer time after the beginning of illumination in the absence of HCO_3^-* than in its presence, causing a lower "steady state" [Q^-] and, therefore, a lower fluorescence yield in "$-HCO_3^-$* chloroplasts" at longer time scales (700 ms–10 s).

In the presence of DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea), which blocks reoxidation of Q^- by $B^{(-)}$ both in the absence and the presence of HCO_3^-*, a faster rise in fluorescence was observed in the absence of HCO_3^-* than in its presence (Stemler & Govindjee 1974). Recently, these results, which were obtained using low light intensities, have been confirmed by the present authors at high light intensity (see Vermaas 1981, Vermaas & Govindjee 1982). We have shown that this effect is not on the oxygen-evolving site as it persists in thylakoids in which the oxygen-evolving site is inactivated by heat treatment and where water is replaced by an artificial donor. The exact location of this additional bicarbonate effect is not yet known, but it appears to be somewhere between Z and Q. The size of this effect is about a factor of 2 (Stemler & Govindjee 1974, Vermaas 1981), and is small compared to the 10–20 fold bicarbonate effects normally observed on electron transport rates between Q and PQ (Govindjee et al. 1976).

In agreement with a major site for the bicarbonate effect not to be on the oxygen-evolving system is the observation that artificial electron donors for PS II, like hydroquinone/ascorbate, $MnCl_2$, NH_2OH, and DPC, are unable to change the $-HCO_3^-$* fluorescence characteristics into $+HCO_3^-$* characteristics in CO_2-depleted chloroplasts in which the O_2-evolving site is inactivated by alkaline Tris washing (Wydrzynski & Govindjee 1975). These results suggest HCO_3^-* action to be between the site of electron donation by the above-mentioned donors and PQ. Since NH_2OH seems to be able to feed electrons almost directly to the PS II reaction center (Bennoun & Joliot 1969, Mohanty et al. 1971, den Haan et al. 1976) (see figure 1), it is probable that the "large" bicarbonate effect is not on the re-reduction of $P680^+$ or Z^+; this is in agreement with conclusions from (a) fluorescence experiments in the μs-domain after flashes (Jursinic et al. 1976) [(the rise in fluorescence yield on this time scale 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 & Butler 1972, Butler et al. 1973)], and (b) the decay kinetics of EPR signal II_{vf} (Jursinic et al. 1976), which monitors the re-reduction of Z^+ by electrons from H_2O (Blankenship et al. 1975). Both the rise in fluorescence yield and the EPR II_{vf} decay kinetics were not influenced significantly by the presence or absence of HCO_3^-*. Thus, HCO_3^-* does not influence the electron flow from H_2O to Z^+ and from Z to $P680^+$. We caution that some care should be taken in interpreting the μs fluorescence yield results as there is also a very fast component of $P680^+$ reduction, with a half-time of 30 ns (van Best & Mathis 1978), in dark-adapted thylakoids. However, using repetitive flashes, under the conditions in which Jursinic et al. (1976) obtained their μs fluorescence results, this very fast component does not seem to be present (Renger et al. 1978, Sonneveld et al. 1979).

The maximal fluorescence yield in CO_2-depleted chloroplasts is not significantly changed by the addition of HCO_3^- (Stemler & Govindjee 1974). Since this yield reflects the total concentration of Q^-, formed by charge separation: $P680.\ Q \xrightarrow{h\nu} P680^+.\ Q^-$, the above result indicates that the number of reaction centres that is able to undergo charge separation is not influenced by HCO_3^-*. This is in apparent contradiction

to the conclusion that the reaction center of PS II is inactivated in CO_2-depleted chloroplasts, the latter was based on the following experiments in which repetitive μs flashes with ~1 sec dark times between the flashes were used: (a) the total oxygen yield is nearly two times larger in the presence than in the absence of HCO_3^{-*} (Stemler et al. 1974); (b) the amplitude of the EPR signal II_{vf} (measuring the concentration of Z^+) is 40% lower in the absence than in the presence of HCO_3^{-*}—this was interpreted to be due to the lowered concentration of $P680^+$, which leads to a lowered concentration of Z^+ (Jursinic et al. 1976); and (c) the amplitude of an absorbance change at 320 nm, which measures the concentration of Q^-, is also 40% lower in the absence than in the presence of HCO_3^{-*}; this was again interpreted to be due to the lowered concentration of active P680 due to partial reversible inactivation of the reaction centre of PS II (Siggel et al. 1977). There are two possibilities to explain the above results: first, it is feasible that a charge separation by a flash is less probable in the absence than in the presence of HCO_3^- due to a HCO_3^{-*} effect at P680 or pheophytin (Pheo, the intermediate that precedes Q, see figure 1). However, this is inconsistent with the above-mentioned fluorescence induction kinetics in DCMU-treated chloroplasts (Stemler & Govindjee 1974, Vermaas 1981). Second, the apparent inactivation of P680 might be due to an effect on the redox state of Q. If, in repeated flash experiments, some Q molecules remain in the reduced state before the next flash, no charge separation can occur between P680 and Q, and, for that reason, $Pheo^-$ and Z will compete to reduce $P680^+$ in those centers. If $Pheo^-$ reduces $P680^+$, no Z^+ will be formed, and no EPR II_{vf} signal will be observed. This will explain the reduced amplitude of flash-induced O_2-evolution, $[Z^+]$ and $[Q^-]$. It is not known why it takes a comparatively long time (~ 20 min) to dark-adapt CO_2-depleted chloroplasts both in the presence and the absence of DCMU (Stemler & Govindjee 1974); furthermore, in the absence of HCO_3^{-*} the reoxidation of Q^- has a very slow component with a half-time of 1 s (Jursinic & Stemler 1981). Therefore, we favour the second possibility, i.e., a part of the Q population is not reoxidized before exposure to the next flash, which explains, as noted above, the data of Jursinic et al. (1976), Stemler et al. (1974) and Siggel et al. (1977).

In this discussion, so far, we have assumed that both Q→B and B→PQ electron transport are slowed down in the absence of HCO_3^{-*}. Strong evidence for this assumption was given by Govindjee et al. (1976)—these authors measured the chl *a* fluorescence decay as a function of flash number: flashes were given 30–50 ms apart and the fluorescence intensity was measured 160 ms after the last flash. In dark-adapted CO_2-depleted chloroplasts, the fluorescence decay is fast after the first two flashes, but it is slowed down after the third and the succeeding flashes (figure 3). These results have been confirmed recently by Jursinic (personal communication). Assuming that a significant proportion of Q^- is reoxidized in the dark time between the flashes (a certain portion will certainly not be reoxidized in the absence of HCO_3^{-*}; Jursinic & Stemler 1981), we can conclude that the reoxidation of B^{2-}, formed after two flashes, is slowed down markedly in the absence of HCO_3^-. [Q^- can transfer its electron only to B and B^-, but not to B^{2-}; if the lifetime of B^{2-} is greatly increased after CO_2-depletion, then the lifetime of Q^- when B^{2-} is present (i.e. after the 3rd and succeeding flashes) will be greatly increased as well; this causes the existence of a high level of fluorescence for a long time.] Addition of HCO_3^- results in a fast fluorescence decay after all the flashes, showing that B^{2-} oxidation can be restored by HCO_3^- addition. The above interpretation

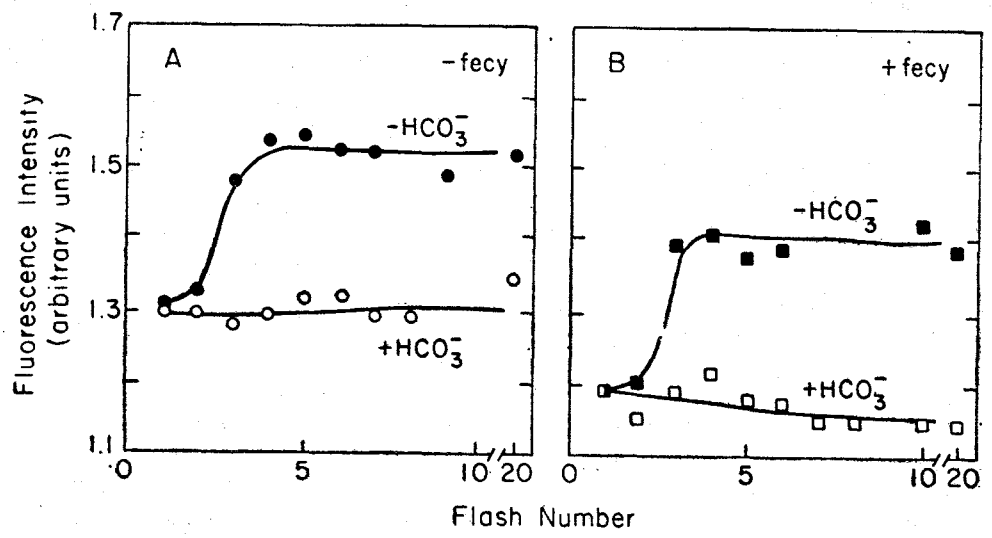

Figure 3 Intensity of chlorophyll *a* fluorescence 160 ms after the last of a series of 3μs saturating flashes, spaced at ~30 ms, as a function of the number of flashes in CO_2-depleted chloroplasts with or without 20 mM $NaHCO_3$. Addition of 20 mM ferricyanide, as indicated. 20μg chl ml^{-1}. The reaction mixture contained 50 mM Na phosphate, 100 mM NaCl, 100 mM Na formate (*p*H=6.8). (*From Govindjee et al. 1976.*)

of the CO_2 effect was confirmed by experiments in which DCMU was added after a flash, followed by fluorescence intensity measurements. DCMU is known to cause a shift in the $Q^-B \rightleftharpoons QB^-$ and $Q^-B^- \rightleftharpoons QB^{2-}$ equilibrium to the left (Velthuys & Amesz 1974); this provides an indirect means to measure the $B/(B^-+B^{2-})$ ratio by fluorescence techniques. In the presence of HCO_3^-*, a periodicity of 2 is observed in a plot of the fluorescence yield after the addition of DCMU as a function of the number of preilluminating flashes—fluorescence is high after the 1st and 3rd and low after the 2nd and 4th flash; however, in the absence of HCO_3^-* this oscillation disappears (figure 4). Since in the absence of HCO_3^-*, QB^{2-}, formed after the second flash, transfers electrons very slowly, addition of DCMU produces Q^-B^- and fluorescence is high, just as after the first flash. Thus, the oscillation disappears.

All experimental results, thus far, can be explained by the suggestion that removal of HCO_3^-* from its site causes: (1) a major (90–100%) inhibitory effect on the electron transport between Q and the plastoquinone pool, and (2) a rather small effect on the electron transport between Z and Q (in the presence of DCMU).

II.A.2. *Delayed light emission (DLE) measurements*

Delayed light emission (DLE), having the same emission spectrum as chlorophyll *a* fluorescence, is assumed to originate from the back reaction of the primary photochemical reaction of photosystem II: $P680^+.\ Q^- \rightarrow P680.\ Q+h\nu$ (Lavorel 1975, Malkin 1977, Amesz & van Gorkom 1978, Govindjee & Jursinic 1979). In DLE measurements, samples are first illuminated to initiate the primary charge separation:

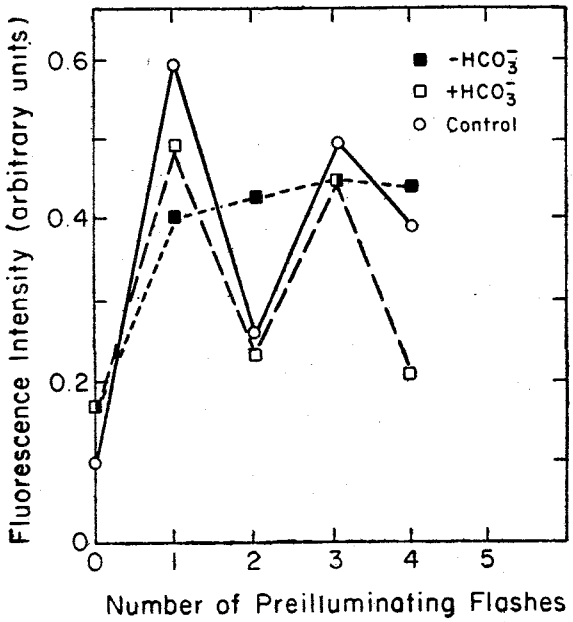

Figure 4 DCMU-induced increase in chlorophyll *a* fluorescence as a function of the number of preilluminating flashes. 20μg chl ml^{-1} (from spinach) o: nondepleted (control) chloroplasts; ■: CO_2-depleted chloroplasts; □: CO_2-depleted chloroplasts+20 mM $NaHCO_3$. Reaction medium as described in the legend of figure 3 (*From Govindjee et al. 1976.*)

P680. $Q \xrightarrow{h\nu} P680^+$. Q^-, which may or may not be followed by the stabilization of charges on neighbouring molecules; the exciting light isturned off and DLE is measured as a function of time in darkness.

Two different types of DLE experiments were performed using CO_2-depleted chloroplasts: (a) on a long time-scale (seconds) (Stemler & Govindjee 1974) and (b) on a short timescale (μs) (Jursinic et al. 1976). (a) *long timescale*: CO_2-depleted chloroplasts show more DLE in the seconds range in the presence of HCO_3^- than in its absence, the presence of DCMU enhances the observed difference in DLE between + and $-HCO_3^-$ samples. If DCMU is injected in the dark just after illumination, then a burst of DLE is observed in samples to which HCO_3^- is added, but not in samples without HCO_3^-. The burst of DLE after the DCMU addition is easily explained by assuming that DCMU shifts $Q^- B \rightleftharpoons Q B^-$ to the left (Velthuys & Amesz 1974); it is not clear, however, why the absence of HCO_3^{-*} inhibits such a burst. One possibility might be that the $QB^- \rightarrow Q^- B$ reaction is slowed down enormously by the absence of HCO_3^{-*}, while another possibility is that the greater portion of back reaction has already occurred in the absence of HCO_3^{-*} before DCMU is injected. This long-range DLE remains difficult to interpret. Parallel measurements on various other reactions on this time-scale are needed before firm conclusions can be made. (b) *short timescale*: DLE in the microsecond range is probably due to a direct back reaction between $P680^+$ and Q^- (Lavorel 1969, 1975, Jursinic & Govindjee 1977). In the absence of HCO_3^{-*}, the kinetics of DLE decay are slowed down compared to that in the presence of HCO_3^{-*}. This means that the Q^- lifetime is longer in the absence of HCO_3^{-*}, which indicates a block after Q (Jursinic et al. 1976).

II.A.3 *Measurements of absorption changes due to quinones*

Transitions in redox state of the quinones can be monitored by absorption spectroscopy. Flash-induced absorption changes at 320 and 334 nm are ascribed to the oxidation or the reduction of the quinone Q (Stiehl & Witt 1969, Witt 1973, Renger 1976, Siggel et al. 1977), while changes in absorption at 265 nm indicate changes in the Q, B and PQ pool (van Gorkom 1974, Klingenberg et al. 1962, Stiehl & Witt 1968, Haehnel 1976, Siggel et al. 1977).

The amplitude of the flash-induced absorption change at 334 nm was observed to be smaller in the absence than in the presence of HCO_3^{-*} (Siggel et al. 1977). This was

interpreted to suggest that a part of the PS II reaction centers was reversibly inactivated by CO_2-depletion. However, these experiments were performed using repetitive flashes, with 200 ms darktime between the flashes, and therefore this decrease in amplitude can be explained by the slower decay of Q^- in the absence of HCO_3^-, as pointed out by Jursinic and Stemler (1981). Furthermore, the decay kinetics of this 334 nm absorption change were different in the absence and the presence of HCO_3^-. The decay of this absorption change observed with the CO_2-depleted samples is much more biphasic than that with CO_2-sufficient ones: in the absence of CO_2, two exponential phases of about equal magnitude with half-life time of 500 μs and 7 ms were observed. In the presence of CO_2, 88% of the decay was fast (450 μs) and the other 12% slow. This slow component was attributed to the formation of PQH_2 or the reduction of oxidized P700. It was suggested that 50% of the CO_2-depleted sample was unaffected in its decay kinetics by the procedure of CO_2 depletion, while the other 50% had slower kinetics, implying a blockage in the reoxidation of Q^- (Siggel et al. 1977). The half-time ($t_{1/2}$) of Q^- oxidation in the absence of HCO_3^-* is about 7 ms; it is slightly slower than measured earlier by the fluorescence method (~3 ms) (Jursinic et al. 1976), and is somewhat faster than the rate measured by Stemler et al. (1974). Simultaneous measurements of flash-induced absorbance change decay at 265 nm (monitoring mainly oxidation of B^{2-} and PQ^{2-}) and at 703 nm (monitoring the $P700^+$ rereduction) show that the predominant phase in the absence of HCO_3^-* is nearly 10-fold slower ($t_{1/2}$ 200 ms) than in its presence (25 ms). The dark decay of the absorbance change at 265 nm in CO_2-depleted samples is interpreted, in accordance with other experimental data, to be controlled by the oxidation of B^{2-} by PQ whereas that in CO_2-sufficient samples by the oxidation of PQ^{2-} (Siggel et al. 1977). Therefore, both Q→B and B^{2-}→PQ electron transport are slowed down by an order of magnitude upon removal of HCO_3^-* from thylakoids; this conclusion is in agreement with the data presented in previous sections. The dark rereduction kinetics of $P700^+$, formed in the light, confirmed that the reactions measured were in the main path of electron flow.

II.A.4. *Measurements of light-induced proton-uptake and release by the thylakoid membrane*

Proton-uptake and release by thylakoid membranes can be monitored by spectrophotometry in an accurate and elegant manner using indicator dyes and appropriate buffers (Junge & Ausländer 1973, Junge et al. 1979).

When flash-induced *p*H changes in the internal space of the thylakoid membrane were measured in CO_2-depleted chloroplasts, then the rapid *p*H change due to proton extrusion by the oxygen-evolving system was decreased compared to control chloroplasts, while a slow component ($t_{1/2}$~100 ms), present in control chloroplasts, was found to disappear (Khanna et al. 1980). This slow component is attributed to the oxidation of PQH_2, which causes proton movement to the inside (Ausländer & 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). This is in apparent disagreement with the idea that cyclic electron transport around PS I, which will involve the PQ pool, is not affected by HCO_3^-* (Radmer & Ollinger 1980), but it can be explained by the assumption that in broken chloroplasts the cyclic phosphorylation is inactivated. The decrease in amplitude of the rapid phase (due to H^+ extrusion in the intrathylakoidal space) was interpreted as indicating an inactivation of PS II

(Khanna et al. 1980). This may be 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 flashes was long enough (10 s) to obtain full recovery of Q. The reversibility could not be tested, however, because the addition of high bicarbonate concentrations to the CO_2-depleted chloroplasts buffered away all *p*H changes. (New experiments are planned by the authors in which the reversibility will be tested by using the latter samples after they have been resuspended in CO_2-free buffer.)

Flash-induced *p*H changes in the outer phase were measured as well (Khanna et al. 1980, see figure 5). No significant alkalinization of the outer phase, due to proton-uptake by the PQ pool, was observed in CO_2-depleted chloroplasts, this could be easily explained by a slowing down of electron transport from B^{2-} to PQ. (An acidification due to the release of internal protons to the exterior of the thylakoids was, however, noticed.)

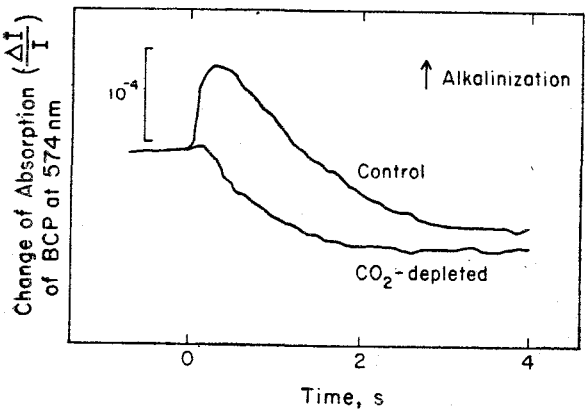

Figure 5 Absorption changes of bromocresol purple (BCP) at 574 nm induced by a single turnover flash in control and CO_2-depleted spinach thylakoids. The reaction mixture contained 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM FeCy, 20 μM bromocresol purple, *p*H=6.4; 10μg chl ml^{-1}. Signal was averaged over 10 flashes ($t_{1/2}$=15μs); dark time between flashes was 10 s. (*From Khanna et al. 1980.*)

The above data indicate that the PQ pool does not make a significant amount of turnovers in the absence of HCO_3^-*. Furthermore, the rate constant for proton leakage in CO_2-depleted thylakoids is increased by a factor of two (k=1.46s^{-1} as compared to k=0.73s^{-1} in control) (Khanna et al. 1980).

II.A.5. *Measurements of oxygen evolution as a function of flash number at high redox potential*

Oxygen evolution per flash in a series of μs flashes, spaced ~ 1 s apart, oscillate with a period of 4, indicating that a charge accumulator exists on the water side of PS II. For a background concerning the mechanism of O_2 evolution, see Radmer and Cheniae (1977) and Govindjee (1980).

Normally, addition of ferricyanide (FeCy), which causes a considerable increase in redox potential in the system, to dark-adapted chloroplasts leads to a significant increase in the oxygen yield in the second flash (Kok et al. 1974); this reflects the ability of these chloroplasts to undergo a double turnover of the system II trap within the duration of the flash. This FeCy-induced double hit is explained by the existence of a component, called C400, which, in its oxidized form, can be reduced rapidly by Q^-. The midpoint potential of this component is around +400 mV (Bowes & Crofts 1980). However, in CO_2-depleted chloroplasts this large double hit probability is absent as the oxygen evolution after the second flash is very low compared to that after the third flash (Radmer & Ollinger 1980). This cannot be ascribed only to a higher probability of misses, α, which is normally observed in CO_2-depleted chloroplasts. For that reason, it was proposed that the electron transport from Q^- to C400 is blocked in the absence of HCO_3^-* (Radmer & Ollinger 1980).

II.A.6. *Studies on HCO_3^-* interaction with PS II herbicides*

Since herbicides like DCMU, atrazine and DNOC (4, 6-dinitro-o-cresol) block electron transport between Q and B (see e.g. Trebst & Draber 1979), it is interesting to investigate if there is a relationship between the binding sites of HCO_3^-* and the herbicides. Stemler (1977) showed that the removal of bound $H^{14}CO_3^-$* by washing with silicomolybdate (SiMo) was partly prevented by prior addition of DCMU. This suggested a close spatial relationship between the DCMU and HCO_3^--binding sites.

A good method to investigate the HCO_3^-*/herbicide interaction more precisely is to compare Hill reaction rates, in the absence and presence of herbicide, after the addition of different concentrations of HCO_3^- to CO_2-depleted chloroplasts. This comparison is made by examining Lineweaver-Burk (LB) plots ($1/v_{Hill}$ versus $1/[HCO_3^-]$, in which v_{Hill} is the Hill reaction rate) under the two conditions. In experiments in which the phenolic herbicide DNOC was used, the two lines (figure 6) in the LB plot intersect at the y-axis indicating a full competition (Dixon & Webb 1964). On the other hand, when DCMU was used, the two lines intersect between the x- and y-axis indicating that DCMU and HCO_3^-* are partially competitive (figure 7) (van Rensen & Vermaas 1981).

Simeton, an atrazine-like herbicide, also shows a partial competition with HCO_3^-* (van Rensen & Vermaas 1981). However, Khanna et al. (1981), who investigated the relationship between HCO_3^-* and atrazine by measuring ^{14}C-atrazine binding in CO_2-depleted chloroplasts with and without HCO_3^- addition, showed a lower atrazine binding in the absence of HCO_3^-*; although these data show clearly that the binding of HCO_3^-* affects the binding of atrazine, and

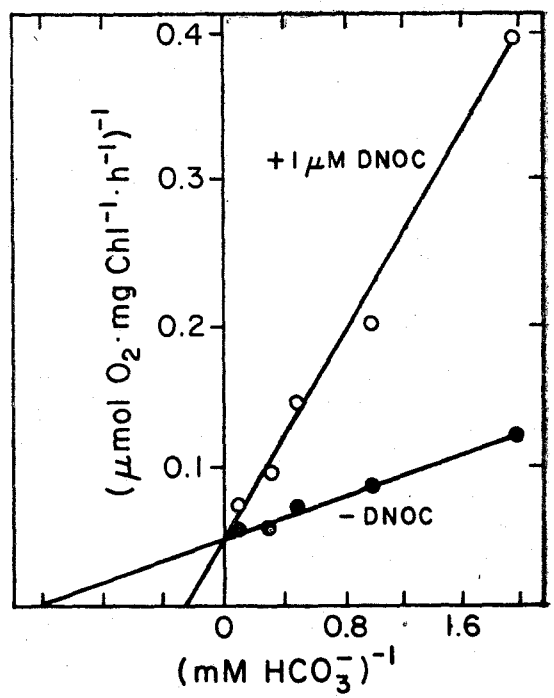

Figure 6 Double reciprocal plot of the Hill reaction rate as a function of the bicarbonate concentration in CO_2-depleted pea chloroplasts in the absence and the presence of 1 μM DNOC. The reaction medium contained 50 mM Na-phosphate, 100 mM Na-formate, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM FeCy, *p*H=6.5. 33 μg chl ml^{-1} *(From van Rensen and Vermaas 1981.)*

that the binding sites are close together, they are in the opposite direction compared to the (partial) competition between the herbicide and HCO_3^-* observed by van Rensen and Vermaas (1981). The reason(s) for this possible discrepancy is (are) not yet known. However, it is clear from all of the above data that the herbicide and HCO_3^-*-binding sites have to be in close proximity. Since the PS II herbicides are known to block electron-transport between Q and B, presumably by changing the redox potential of B (see, e.g., Arntzen et al. 1981), it is not at all surprising that HCO_3^-* should have its major effect near B.

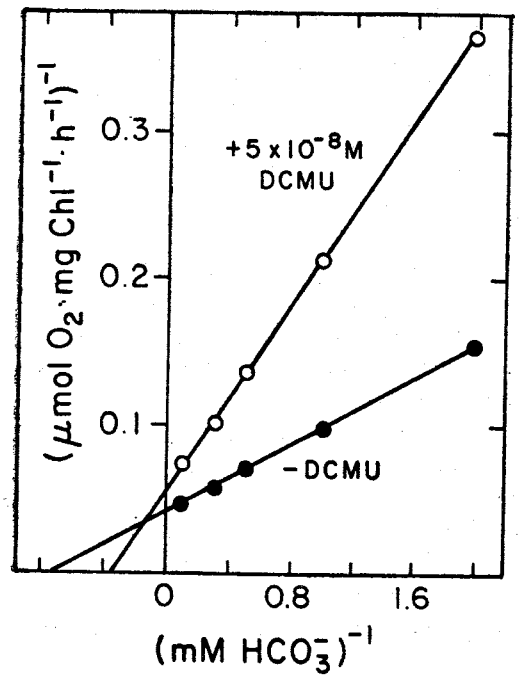

Figure 7 Double reciprocal plot of the Hill reaction rate as a function of the bicarbonate concentration in CO_2-depleted pea chloroplasts in the absence and the presence of 5×10^{-8} M DCMU. Reaction medium as in figure 6
(*From van Rensen and Vermaas 1981.*)

II.A.7. *Observations on HCO_3^-*-binding with a periodicity of two*

When dark-adapted CO_2-depleted chloroplasts are given a certain number of flashes, $H^{14}CO_3^-$ is added, and binding monitored, ^{14}C-binding to the site appears to be dependent on flash number: after one flash, there is significantly less binding than after darkness, while after two or more flashes a larger amount of ^{14}C is bound than after the first flash (Stemler 1979). The oscillation in binding seems to dampen out rather fast and the amplitude of the oscillation is small (~15%). Furthermore, the percentage recovery of Hill reaction—10 seconds compared to 2.5 min after HCO_3^- addition—is lower after one flash than after two flashes or without flashes (figure 8). This might indicate that HCO_3^-* cannot bind to the site if B is in the B^- or B^{2-} form; a direct interaction of HCO_3^-* and B was proposed (Stemler 1979). This may explain why a dark period helps in the reactivation of CO_2-depleted chloroplasts by HCO_3^- addition; however, if formate, which is normally used in the suspension medium for CO_2-depleted chloroplasts to keep the

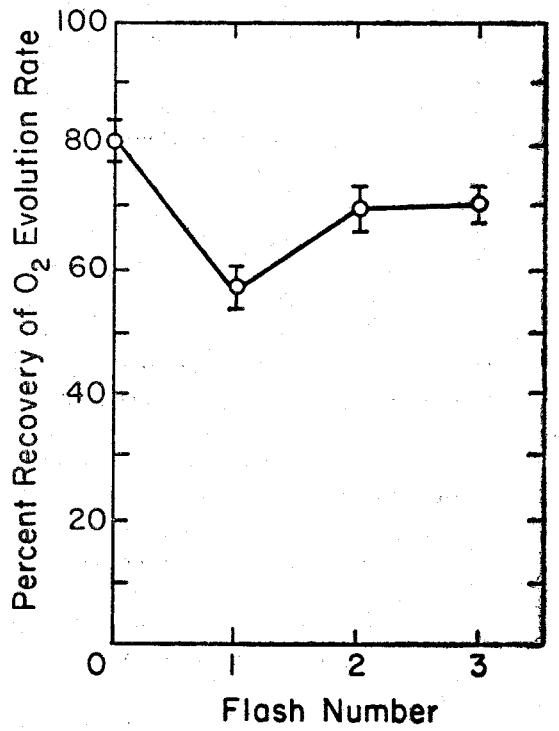

Figure 8 Restoration of oxygen evolution as a function of flash number prior to the addition of HCO_3^-. The CO_2-depleted chloroplasts were subjected to a varying number of saturating light flashes, spaced 1s apart; 10 mM $NaHCO_3$ was added. After 10 s, FeCy (2 mM) was injected and the chloroplasts were given continuous saturating light. After 30 s of illumination, the chloroplasts were allowed to reactivate completely in a subsequent 2.5 min dark period. Then, the FeCy Hill reaction rate was measured again. The percent recovery is the initial slope of the oxygen trace observed during the first period of continuous illumination divided by that observed after complete recovery, multiplied by 100. Each point expresses the mean of at least 5 measurements. The reaction medium contained 100 mM Na phosphate (pH=6.8), 175 mM NaCl, and 100 mM Na formate. 15 μg chl ml^{-1}.
(*From Stemler 1979.*)

atmospheric CO_2 from binding to the active site, was left out, then a rather rapid reactivation was seen after the HCO_3^- addition even in saturating light (Vermaas & van Rensen 1981). Furthermore, Sarojini and Govindjee (1981 a, b) observed a very fast reactivation of CO_2-depleted chloroplasts by the addition of CO_2 or HCO_3^- in the light even in the presence of high concentrations of formate, although this reactivation was faster in the absence of formate and higher rates were obtained with dark incubation (Sarojini & Govindjee, personal communication).

Although a direct specific interaction between HCO_3^-* and B would be very attractive, we feel that the evidence is not yet very convincing.

II.A.8. *Use of different electron acceptors and of trypsin*

In the presence of SiMo (silicomolybdate, $SiMo_{12}O_{40}^{4-}$), an artificial electron-acceptor known to accept electrons from Q (Giaquinta & Dilley 1975, Zilinskas & Govindjee 1975), and DCMU, no significant bicarbonate effect is observed (Khanna et al. 1977, van Rensen & Vermaas 1980). This suggests that there is no major site of HCO_3^-* action before Q, and that the reversible "inactivation" of the PS II reaction centers, as observed by Jursinic et al. (1976) and Siggel et al. (1977), is caused by effects on the reoxidation of Q. However, a large HCO_3^- effect is observed if DAD (2, 3, 5, 6-tetramethylphenylenediamine) in the oxidized form serves as an electron-acceptor and 0.5μM DBMIB (2, 5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) is added to inhibit electron flow beyond the PQ pool (Khanna et al. 1977). These data indicate that the major bicarbonate effect is indeed located between Q and PQ. No measurable effect of HCO_3^-* is observed in a PS I reaction as monitored by electron flow from reduced DAD to methylviologen in the presence of DCMU (Khanna et al. 1977).**

Recently, the influence of trypsin on the FeCy Hill reaction in CO_2-depleted chloroplasts has been investigated; trypsin removes herbicides from their binding sites on the thylakoid membrane, and makes Q accessible to FeCy (Renger 1976, van Rensen & Kramer 1979). Trypsin is able to restore electron transport in CO_2-depleted chloroplasts with FeCy as an electron-acceptor without the addition of HCO_3^- to rates comparable to those after the HCO_3^- addition. This means that no significant bicarbonate effect is observed in the electron-transport chain from H_2O to Q (van Rensen & Vermaas 1981). Independently, Khanna et al. (1981) obtained comparable results: after trypsin incubation, no bicarbonate effect on the FeCy Hill reaction was observed. However, a restoration of the Hill reaction by trypsinisation without HCO_3^- was not seen. A difference in suspension media is the possible cause for this discrepancy.

Using normal (i.e. non-CO_2-depleted) chloroplasts, the DCMU insensitive SiMo Hill reaction is inhibited by high concentrations (~20mM) of HCO_3^- (Crane & Barr 1977, Barr & Crane 1980). This might also indicate a close relationship between the SiMo electron acceptance site and the HCO_3^-*-binding site (Vermaas & van Rensen 1981).

After the present manuscript was submitted for publication, the authors became aware of a recent paper by K Fischer and H Metzner (*Photobiochem. Photobiophys.* **2, 133–140, 1981). These authors report that methylviologen-mediated Mehler reaction (monitoring non cyclic electron transfer through PS II and PS I) in thylakoids treated with hydroxylamine (blocking $P680^+$ reduction by the physiological donor Z and reducing $P680^+$ via an unknown donor D; see figure 1) is relatively insensitive to HCO_3^-*. The interpretation of this result appears to be in contradiction to a large number of experimental observations reviewed in the present paper. For example, Wydrzynski and Govindjee (1975) have observed a large HCO_3^-* effect using NH_2OH as an electron donor. It is not known whether NH_2OH treatment, as done by Fischer and Metzner, has effects other than on the donor side of PS II. The discrepancy remains to be resolved.

II.B. *POSTULATED EFFECTS ON THE OXIDIZING SIDE*

II.B.1. *Effects on the "S" states*

If oxygen evolution in CO_2-depleted chloroplasts is measured as a function of flash number, the damping of the oscillation with period of 4 (Kok et al. 1970) is faster than in CO_2-supplied chloroplasts (Stemler et al. 1974); this may indicate that the miss parameter α, the probability of not undergoing a net change in the S-state after a flash, is higher in the absence of HCO_3^-*. Since the miss parameter can be due to effects on the reaction center itself or be controlled by the reducing side of PS II (Radmer & Cheniae 1977), this result does not imply that it originates from the oxidizing side of PS II.

The dark conversion of the S_n state (created by a light flash) into the S_{n+1} state, measured by varying the dark time between flashes seems to be slower in the absence than in the presence of HCO_3^-*: $S_1' \rightarrow S_2$ and $S_2' \rightarrow S_3$ are extended by a factor of more than 10 (from 0.6 to 10 ms) (Stemler et al. 1974). For example, the $S_1' \rightarrow S_2$ reaction can be written as M^+. $P680^+$. $Q^- \longrightarrow M^{2+}$. P680. Q (here, M is defined as the

$$\overset{\frown}{B \quad B^-}$$

charge accumulator); no further primary charge separation can occur on S_1' as the reaction center is blocked. The relaxation of S_1' to S_2 is usually governed by the electron flow from Q^- to B because the electron flow from Z to $P680^+$ is a much faster process (see Sonneveld et al. 1979). If this explanation is correct, then a longer $S_n' \rightarrow S_{n+1}$ "reaction time" would indeed be expected in the absence of HCO_3^-* because the Q^- lifetime is much longer in this case (Jursinic et al. 1976, Siggel et al. 1977, Jursinic & Stemler 1981). However, the transfer of electrons from H_2O to Z^+ could be as slow as electron transfer from Q^- to B and thus control $S_n' \rightarrow S_{n+1}$ transitions. We do not favour this possibility because electron flow from H_2O to Z^+, as measured by the decay of EPR signal II_{vf}, was independent of HCO_3^-*, and was of the order of 0.8 ms both with and without HCO_3^-* (Jursinic et al. 1976).

Neither the deactivation of the S states (e.g. S_2 and S_3) nor the kinetics of oxygen evolution, as measured by a Joliot electrode, were affected by HCO_3^-* (Stemler et al. 1974). However, at pH=5.3, small differences in the kinetics of oxygen evolution were detected ($t_{1/2}=4.93\pm0.18$, $+HCO_3^-$*; $t_{1/2}=5.55\pm0.27$, $-HCO_3^-$*) (Stemler 1980c). These differences could not be confirmed by the present authors (Vermaas & Govindjee, unpublished). The experimental data described above are easily explained by a decrease in electron transport from Q to PQ.

Sodium formate ($NaHCO_2$), which probably competes with HCO_3^-* (Good, unpublished results), lengthens the relaxation times of $S_2' \rightarrow S_3$ and $S_3' \rightarrow S_0$ at pH=8.2, without affecting $S_0' \rightarrow S_1$, $S_1' \rightarrow S_2$, the miss parameter α, and the double hit paramter β (Stemler 1980 b). Stemler proposed that those S-state transitions which show extended relaxation time, in the presence of formate, must result in momentary release and rebinding of CO_2: formate can occupy the HCO_3^-*-binding site for a moment, resulting in a slowing down of CO_2 rebinding and, therefore, of the relaxation time (Stemler 1980 b). However, at pH=5.3, the $S_0' \rightarrow S_1$ and $S_1' \rightarrow S_2$ are also slowed down by formate (Stemler 1982); this would imply that at this pH formate is able to remove HCO_3^-* from its binding site. Retardation of Q^- reoxidation by removal of HCO_3^-* would explain the above effects. Indeed, bound HCO_3^-* can be removed by washing thylakoids with formate-containing medium at low pH (Stemler 1980 a).

A specific effect on certain S-states, as observed at pH=8.2, however, needs a different explanation. One possibility is

given above (Stemler 1980b), but another possibility is evolved below. Bouges-Bocquet (1981) has suggested that two different electron donors, Z_1 and Z_2, donate electrons to P680 in parallel, and each of them is related to transitions in two S-states: Z_1 is connected to S_0 and S_1, and Z_2 to S_2 and S_3. Formate may slow down the Z_2 reduction but not the reduction of Z_1. This means that this formate effect is not necessarily related to HCO_3^-* binding; this thought is strengthened by the fact that the miss parameter α is not influenced by formate (Stemler 1980 b), as would be expected if HCO_3^-* was not removed from its binding site. It should be emphasized that although formate and HCO_3^-* may compete, additional effects of formate may occur.

II.B.2. *$H^{14}CO_3^-$* binding studies*

The rate of binding of $H^{14}CO_3^-$* to CO_2-depleted chloroplasts is independent of *p*H in the first two minutes, but when the thylakoids are equilibrated at a certain *p*H for 5 min, followed by $H^{14}CO_3^-$ addition, then a much faster binding is observed at *p*H=6.0 than at *p*H=7.8 (figure 9). These results were interpreted by Stemler (1980 a) to suggest that the internal *p*H (which is said to be equal to the external *p*H after 5 min equilibration, but not in the first two minutes) rather than the external *p*H governs

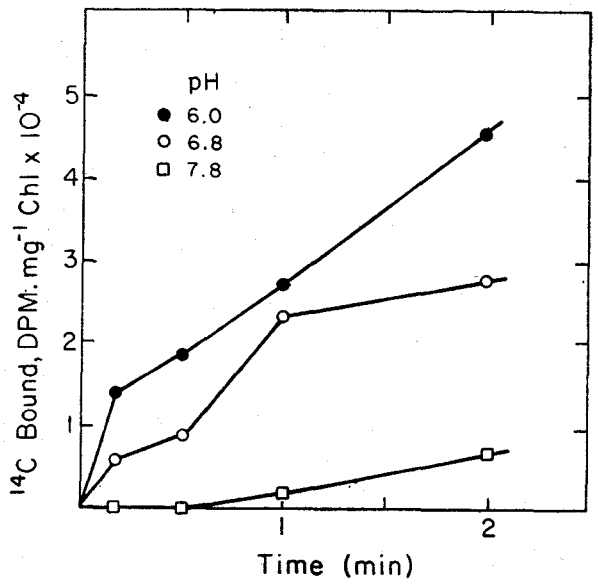

Figure 9 The rate of binding of 0.33 mM $H^{14}CO_3^-$* to CO_2-depleted chloroplasts after 5 minutes equilibration time in reaction mixtures at various *p*H values. Binding of $H^{14}CO_3^-$* was stopped after the indicated times by the addition of enough $NaHCO_3$ (unlabelled) to give a ratio of unlabelled to labelled $NaHCO_3$ of about 300. The reaction medium contained 100 mM Na phosphate, 10 mM NaCl, 400 mM sucrose and 0.1 mM FeCy, at various *p*H values *(From Stemler 1980a.)*

HCO_3^-*-binding. Since the oxygen-evolving site is located on the inner side of the thylakoid membrane (Radmer & Cheniae 1977), it may be possible that there is an interaction of HCO_3^-* with the oxygen-evolving site. However, the CO_2-depleted chloroplasts are uncoupled (Khanna et al. 1977) and, thus, should allow a fast *p*H equilibrium between the inside and the outside of the thylakoid vesicle. Furthermore, even in non-depleted (control) chloroplasts the equilibration is rather rapid (rate constant of proton leakage: $0.73 s^{-1}$) (Khanna et al. 1980). This means that if the internal *p*H is important, differences in $H^{14}CO_3^-$* binding should be lost within a couple of seconds. This is not observed. Therefore, another explanation for these experimental data should be sought.

II.B.3 *Possible involvement of HCO_3^-* in oxygen evolution*

Warburg and Krippahl (1958), the discoverers of the bicarbonate effect, assumed that this effect was related directly to the oxygen evolution: CO_2 was assumed to be the direct precursor of O_2. The ^{18}O labelling experiments by Ruben et al. (1941) indicated that H_2O, and not CO_2, was the source of evolved O_2. This was confirmed by more sophisticated ^{18}O labelling experiments by Stemler and Radmer (1975) and Radmer and Ollinger (1980); therefore, H_2O is the ultimate source of O_2. However, some evidence may point to an involvement of CO_2 as a direct precursor of photosynthetic O_2 (Metzner et al. 1979). Stemler (1980 b) has recently proposed a scheme of photosynthetic O_2 evolution in which CO_2 is bound to a site near Mn which is followed by a release of CO_2 and H^+. We consider this proposal premature in view of the absence of unequivocal data on effects of CO_2 on the O_2-evolving mechanism. The conclusion of Section II of this review concerning the site of action of HCO_3^-* is, in our opinion, that HCO_3^-* is necessary for the efficient electron transport from Q^- to PQ, other possible roles of HCO_3^-* are to slow down slightly the net charge separation between P680 and Q and to allow electron transport from Q^- to C400. Although some experimental evidence may point at a bicarbonate effect on the oxidizing side of PS II, none of the data show this to be on the oxygen-evolving system. Furthermore, there are many criticisms (see above) concerning the view that a CO_2 effect exists on the oxidizing side of PS II. We believe that there is no real evidence for a direct HCO_3^-* action on the oxidizing side of PS II. This is strengthened by the evidence stated in Sections II.A.1 to II.A.8 which do not point to any HCO_3^-* action "before" P680.

III. The "Active species" involved in the Bicarbonate Effect

A problem that has puzzled many investigators is whether HCO_3^- or CO_2 is the species that binds to the specific binding site. Since formate (HCO_2^-, resembling HCO_3^-) facilitates CO_2-depletion (Good 1963) and appears to be an inhibitor for HCO_3^-*-binding (Khanna et al. 1977, N. Good, personal communication), HCO_3^- may be responsible for the bicarbonate effect. At *p*H = 5.8, where the CO_2/HCO_3^- equilibrium is in favour of CO_2, a higher concentration of bicarbonate is needed to restore maximal Hill reaction activity than at *p*H = 6.8, where HCO_3^- prevails ($pK_A = 6.4$ for the reaction: $H_2O + CO_2 \rightleftharpoons HCO_3^- + H^+$) (Stemler & Govindjee 1973); this finding is in apparent agreement with HCO_3^- being the binding species. However, the HCO_3^-* affinity for the binding site is likely to be a function of *p*H, and, thus, no unequivocal conclusion about the "active species" can be made from these data. Detailed measurements on the *p*H dependence of the bicarbonate effect show an optimum around 6.5–6.8 (Khanna et al. 1977, Vermaas

& van Rensen 1981); these *p*H values are close to the pK_A of CO_2 and, thus, CO_2 and HCO_3^- are present in comparable amounts at the optimum of the bicarbonate effect. This observation coud be taken to suggest a function of both CO_2 and HCO_3^- in the bicarbonate effect (Vermaas & van Rensen 1981). Sarojini and Govindjee (1981a) proposed that CO_2 is necessary for at least "the first step" in the bicarbonate effect because reactivation of the FeCy Hill reaction was much faster (<2s) after the addition of CO_2 than after the addition of HCO_3^- (where a lag of 6–8 s was observed) at 5° C. A working hypothesis explaining these data has been recently proposed (Vermaas & van Rensen 1981, figure 10): HCO_3^- binds to a binding site, which is located on the outer part of the

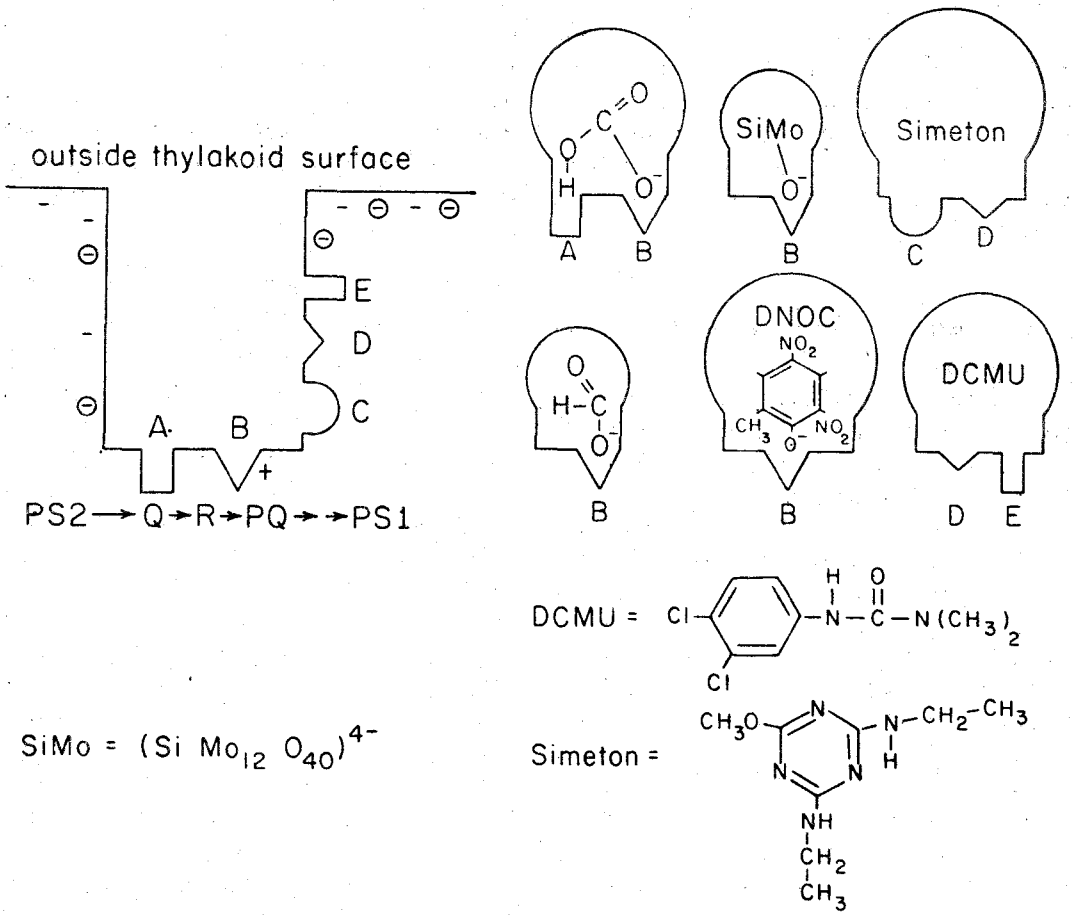

Figure 10 A hypothetical scheme for the bicarbonate-binding site. −: negative charge existing in dark and light; (⊖): negative charge existing in light only. B (not necessarily the intermediate B in figure 1) is a non-specific binding site to which molecules with a $=C-0^-$ group can bind. R, in this diagram, is equivalent to B in figure 1. Electron transport can only proceed when there is also binding to site A. Only HCO_3^- is able to do so. The herbicides DCMU and simeton bind to sites on the pathway from the bulk phase to the HCO_3^- binding site; these herbicides cover part of the HCO_3^--binding site. This causes a change in the affinity of the site for HCO_3^- *(Adapted from Vermaas and van Rensen 1981.)*

membrane close to the herbicide-binding sites, "buried" under negative charges. The negatively-charged surface of the thylakoid membrane becomes even more negatively charged in the light (e.g., see Schapendonk et al. 1980) making it more difficult for HCO_3^- to reach the binding site. However, the uncharged, apolar CO_2 should be able to reach the site without any problem. If formate is present, formate bound to the binding site will not even be able to leave the binding environment (the "pocket") if released from the site because of the repulsion by negative charges. Thus, in the presence of formate and light, no appreciable restoration of the Hill reaction will be observed upon CO_2 addition to CO_2-depleted thylakoids. However, in the absence of formate, CO_2 will be able, after having passed the negative "shield" and after being converted into bicarbonate, to bind to the site without having to compete with formate. Therefore, it will easily restore electron transport. Thus, CO_2 is suggested to be the diffusing species, and HCO_3^- the binding species. This working hypothesis is consistent with the experimental data of Vermaas and van Rensen (1981) and Sarojini and Govindjee (1981a). However, shielding of the negative surface charges with divalent or trivalent cations did not cause a faster reactivation of the Hill reaction by HCO_3^- as would be expected (Sarojini & Govindjee 1981b). Therefore, the working hypothesis seems to be too simple to explain all the experimental data. One possibility to explain why shielding of negative charges on the outer side of the thylakoid membrane does not allow HCO_3^- in the bulk phase to reach the binding site is to assume the existence of another barrier between the outside of the thylakoid membrane and the HCO_3^-*-binding site. This barrier might be in the lipid and/or the hydrophobic protein portion of the membrane; through this barrier CO_2, not HCO_3^-, will be able to diffuse. However, it is not clear how formate ($pK_{A\ HCOOH}=3.75$) will be able to leave the binding site as very few (0.1%) molecules will be in the diffusable uncharged acid form.

The observed *p*H dependence of the bicarbonate effect may also be explained by suggesting another mechanism of HCO_3^-* action: CO_2 binds to a NH_2 group of a protein residue (arginine or lysine) forming a carbamate (see e.g., Gurd et al. 1980, figure 11). Since, in this hypothesis, both CO_2 (requiring a low *p*H for high concentration) and an uncharged amino group (requiring high *p*H) are involved, a bell-shaped curve of *p*H versus carbamate concentration is expected (Gurd et al. 1980); this is in agreement with the experimental data on the *p*H dependence of the bicarbonate effect (Khanna et al. 1977, Vermaas & van Rensen 1981). Such a

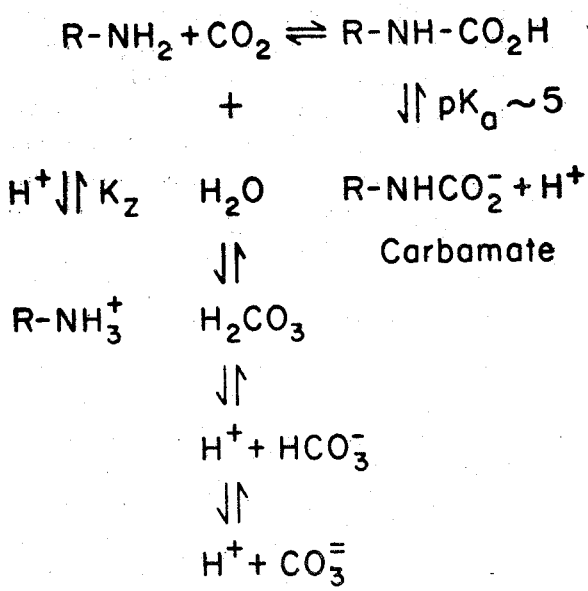

Figure 11 Formation of a carbamate (R-NH-CO_2^-) by reaction of CO_2 with a free amino group on a protein. *R*=Protein; K_z=dissociation constant of R-NH_3^+. (*From Gurd et al. 1980.*)

carbamate formation is known to occur when CO_2 binds to haemoglobin, or when CO_2 binds to activate the ribulose-1, 5-bisphosphate carboxylase (Lorimer & Miziorko 1980).

From the above discussion it is clear that no definite conclusion can be drawn yet about the "active species" involved in the bicarbonate effect. Stemler (1980, a b), who has proposed a HCO_3^-* action on the oxygen -evolving site, suggested that CO_2, not HCO_3^-, is the form that initially binds to the site, and after binding is converted into HCO_3^-. He further suggested that in the light HCO_3^- is converted back into CO_2 which is (at least temporarily) released from the site. Both HCO_3^- and formate were proposed to be competitive inhibitors for CO_2 binding because chloroplasts have lower activity at *p*H=8.0 (where nearly all inorganic carbon is in the HCO_3^- form) with than without the addition of HCO_3^- (50 mM) or HCO_2^- (100 mM) (Stemler 1980 a). However, addition of salts is known to decrease the stability of the Hill reaction (Vermaas & van Rensen, unpublished) and this might have caused the observed effects of HCO_3^- or HCO_2^- addition. Thus, the HCO_3^-* action on the oxidizing side of PS II, if there is any, is mechanistically still not understood and is certainly in a speculative stage.

IV. Conclusions: A possible role of the bicarbonate effect *in vivo*

Although a rather clear picture has emerged concerning the sites of action of HCO_3^-* (a major effect on electron transport from Q^- to PQ; auxiliary effects on electron transport between P680 and Q, and from Q^- to C400, no effects on PS I electron transport), the molecular mechanism of this HCO_3^-* action is still unknown. Further investigations are needed to understand the HCO_3^-* action in its full extent. Although all recent bicarbonate experiments were done with broken chloroplasts, we are sure that the bicarbonate effect is not an "artifact" caused by our experimental conditions. Although we have no positive evidence yet, we believe that this effect must be of significant importance to intact systems as well. It has been observed in intact algae (Warburg & Krippahl 1958), and recently, perhaps, in intact wheat leaves (Gerbaud & André 1980).

To understand what function the bicarbonate effect may have *in vivo*, it is necessary to estimate CO_2 and HCO_3^- concentrations in chloroplasts in the plant. The CO_2 concentration in leaf cells decreases to a very low level when stomata close while CO_2 fixation continues to be active and the leaves reach the compensation point (Black 1973). It is reasonable to assume that the CO_2 concentration near the thylakoids, under the above-mentioned conditions, is equal to or lower than the compensation point. In C_3 plants, compensation points of 30 to 70 ppm CO_2 have been measured (Black 1973) which correspond to 0.9 to 2.1 μM dissolved CO_2. It has been estimated that more than 5 μM HCO_3^-* saturates the "bicarbonate effect" in thylakoids in the absence of "formate-like" anions (Vermaas & van Rensen 1980). This value is higher than the CO_2 concentration at the compensation point (1.4 μM CO_2 for 50 ppm CO_2). The HCO_3^- concentration at the compensation point will always be a function of $[CO_2]$ and *p*H. In the light, the *p*H in the stroma is approximately 8, while in the intrathylakoidal space it is only about 5 (Werdan et al. 1975). The thylakoid membrane surface is negatively charged (Schapendonk et al. 1980), and, therefore, the *p*H close to the thylakoid surface will be significantly lower than in the stroma. No measurement of local *p*H values at specific sites on the outside of the thylakoid membrane is available. If we assume a *p*H of 6.5 at the HCO_3^-*-binding site, CO_2 and

HCO_3^- are in nearly equimolar concentrations (pK_A=6.4), and, therefore, [HCO_3^-] near the binding site is about 1.5 μM Whichever is the binding species (HCO_3^- or CO_2), [HCO_3^{-*}] near the binding site under compensation point conditions is lower than the saturating concentration. Therefore, our belief that the bicarbonate effect is of physiological significance has a reasonable basis.

Why do plants prefer to "turn off" photosynthetic electron transport before CO_2-fixation stops? The following hypotheses for the bicarbonate effect in vivo are presented for consideration: (a) When CO_2 is exhausted, NADPH accumulates as it cannot be consumed in the conversion of CO_2 into carbohydrates. Consequently, [$NADP^+$] is lowered; this increases the chance that another acceptor, e.g. O_2, will accept electrons from the reducing end of the electron transport chain (see figure 1). If O_2 is used as an electron acceptor, then not only H_2O_2 will be formed, but also the superoxide radical anion O_2^-; the latter might destroy some chloroplast functions and thus the whole plant. Since the absence of HCO_3^{-*} blocks non cyclic electron transport, the above scenario may not take place at all. (b) The absence of CO_2 in whole plants may result in an accumulation of NADPH and NADH, and the depletion of $NADP^+$ and NAD^+. It is known that NAD(P)H and $NAD(P)^+$ are necessary for several biochemical pathways. Thus, a large shift in the ([NADPH]+[NADH])/([$NADP^+$] + [NAD^+]) ratio may cause a distortion of equilibria resulting in an accumulation of unwanted, and possibly harmful, intermediates (Vermaas & van Rensen 1980).

In our opinion, a thorough understanding of the bicarbonate effect in a relatively simple system, the thylakoid membranes, would greatly help us in understanding such effects in the intact plant which is, indeed, a rather complex system.

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