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Minireview/hypothesis

The molecular mechanism of the Bicarbonate effect at the Plastoquinone reductase site of Photosynthesis

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Abstract. It has been known for some time that bicarbonate reverses the inhibition, by formate under HCO_3^--depletion conditions, of electron transport in thylakoid membranes. It has been shown that the major effect is on the electron acceptor side of photosystem II, at the site of plastoquinone reduction. After presenting a historical introduction, and a minireview of the bicarbonate effect, we present a hypothesis on how HCO_3^- functions in vivo as (a) a proton donor to the plastoquinone reductase site in the D1-D2 protein; and (b) a ligand to Fe^{2+} in the Q_A-Fe-Q_B complex that keeps the D1-D2 proteins in their proper functional conformation. They key points of the hypothesis are: (1) HCO_3^- forms a salt bridge between Fe^{2+} and the D2 protein. The carboxyl group of HCO_3^- is a bidentate ligand to Fe^{2+}, while the hydroxyl group H-bonds to a protein residue. (2) A second HCO_3^- is involved in protonating a histidine near the Q_B site to stabilize the negative charge on Q_B. HCO_3^- provides a rapidly available source of H^+ for this purpose. (3) After donation of a H^+, CO_3^{2-} is replaced by another HCO_3^-. The high pKa of CO_3^{2-} ensures rapid reprotonation from the bulk phase. (4) An intramembrane pool of HCO_3^- is in equilibrium with a large number of low affinity sites. This pool is a H^+ buffering domain functionally connecting the external bulk phase with the quinones. The low affinity sites buffer the intrathylakoid [HCO_3^-] against fluctuations in the intracellular CO_2. (5) Low pH and high ionic strength are suggested to disrupt the HCO_3^- salt bridge between Fe^{2+} and D_2. The resulting conformational change exposes the intramembrane HCO_3^- pool and low affinity sites to the bulk phase.

Two contrasting hypotheses for the action of formate are: (a) it functions to remove bicarbonate, and the low electron transport left in such samples is due to the left-over (or endogenous) bicarbonate in the system; or (b) bicarbonate is less of an inhibitor and so appears to relieve the inhibition by formate. Hypothesis (a) implies that HCO_3^- is an essential requirement for electron transport through the plastoquinones (bound plastoquinones Q_A and Q_B and the plastoquinone pool) of photosystem II. Hypothesis (b) implies that HCO_3^- does not play any significant role in vivo. Our conclusion is that hypothesis (a) is correct and HCO_3^- is an essential requirement for electron transport on the electron acceptor side of PS II. This is based on several observations: (i) since HCO_3^-, not CO_2, is the active species involved (Blubaugh and Govindjee 1986), the calculated concentration of this species (220 μM at pH 8, pH of the stroma) is much higher than the calculated dissociation constant (Kd) of 35–60 μM; thus, the likelihood of bound HCO_3^- in ambient air is high; (ii) studies on HCO_3^- effect in thylakoid samples with different chlorophyll concentrations suggest that the "left-over" (or "endogenous") electron flow in bicarbonate-depleted chloroplasts is due to "left-over" (or endogenous) HCO_3^- remaining bound to the system (Blubaugh 1987).

Abbreviations: DCMU – 3-(3,4-dichlorophenyl)–1, 1-dimethylurea (common name: diuron), PSII – photosystem II, Q_A – first plastoquinone electron acceptor of PSII, Q_B – second plastoquinone acceptor of PS II.

Introduction

Photosynthesis involves the oxidation of H_2O and the reduction of CO_2. The energy required for this reaction is supplied by the light that is absorbed by the photosynthesis pigments and transferred with great efficiency to the reaction center chlorophylls (see e.g. Pearlstein 1982, van Grondelle and Amesz 1986). In the early days of photosynthesis research, it was not known whether the O_2 that is evolved came from H_2O or from CO_2. From the overall chemical equation for photosynthesis

$$H_2O + CO_2 + \text{light} \rightarrow O_2 + 1/n\ (CH_2O)_n \quad (1)$$

it was supposed by most early workers that CO_2 was the source of evolved O_2, with the chlorophyll somehow catalyzing the transfer of carbon from CO_2 to H_2O. An example of this was the Willstätter-Stoll hypothesis, which had hydrated CO_2 reacting with chlorophyll to yield formaldehyde and O_2, with the formaldehyde then undergoing enzymatic condensation in the conversion to carbohydrates (for an excellent description of this hypothesis and its subsequent loss of favor, see van Niel (1949)). It is now firmly established that the evolved O_2 comes from H_2O (for a historical precedence, see Wurmser (1987)). However, before introducing the role of bicarbonate in photosystem II (PS II), we review the major lines of evidence for H_2O as the substrate for O_2 evolution, since some controversy over this question has continued to the present day, fueled by the requirement for bicarbonate in PS II.

The first major line of evidence for H_2O as the source of photosynthetic O_2 came from comparative studies between the green and purple sulfur-reducing bacteria and green plants (van Niel 1931, also see van Niel 1941, Wraight 1982, Wurmser 1987). In these bacteria, the overall reaction for photosynthesis is

$$2\ H_2S + CO_2 + \text{light} \rightarrow 2\ S + 1/n\ (CH_2O)_n + H_2O \quad (2)$$

Eqn. 1 from green plants can be rewritten as

$$2\ H_2O + CO_2 + \text{light} \rightarrow O_2 + 1/n\ (CH_2O) + H_2O \quad (3)$$

The similarity between Eqns. 2 and 3 is obvious; this led van Niel to propose that photosynthesis is a light-catalyzed oxidation-reduction reaction of the form

$$2\ H_2A + CO_2 + \text{light} \rightarrow 2\ A \text{ or } A_2 + 1/n\ (CH_2O)_n + H_2O \qquad (4)$$

In the green and purple photosynthetic bacteria A would be sulphur, whereas in higher plants, algae and cyanobacteria it would be oxygen. Thus, the implication was clear that the source of photosynthetically derived O_2 is H_2O and not CO_2.

This generalized equation for photosynthesis gained strong support with the demonstration that a variety of compounds can be photosynthetically oxidized by the purple sulfur bacteria: elemental sulfur, sulfite and thiosulfate can all be oxidized to sulfate (van Niel 1931); elemental selenium is oxidized to selenate (Saposhnikov 1937, cited by van Niel 1941); various organic substances can be used as hydrogen donors (c.f. Foster 1940); and some species can use molecular hydrogen (c.f. French 1937). Only when H_2O is the hydrogen donor is O_2 evolved. Thus, plant photosynthesis as a special case of the generalized equation became widely accepted after the pioneering work of van Niel (also see Wurmser 1987).

Nevertheless, it could be argued that what occurs in photosynthetic bacteria is not necessarily what occurs in higher plants, algae and cyanobacteria. Metzner (1975), for example, argues that since plants (including cyanobacteria), unlike the photosynthetic bacteria, require two photosystems to transfer electrons from the primary donor to the terminal acceptor, that it is not valid to compare them. Furthermore, the bacterial photosystem requires electron sources that are relatively easy to oxidize, compared to the O_2-generating PS II. On the other hand, Gaffron (1940) succeeded in adapting cultures of the green alga *Scenedesmus* to the utilization of H_2, so that they photosynthesized without evolution of O_2. The H_2/CO_2 quotient was the same as in purple photosynthetic bacteria. Thus, at least in these species, oxygenic photosynthesis seems to fit the generalized equation of van Niel. Nevertheless, the hypothesis that O_2 comes from H_2O needed to be tested. The test became possible with the availability of oxygen isotopes.

The isotopic composition of the evolved O_2 from photosynthesizing *Chlorella* cells that were supplied with H_2O or HCO_3^- enriched in ^{18}O matched that of the water, not of the HCO_3^-; thus, it was concluded that the O_2 came from the H_2O (Ruben et al. 1941). Such experiments are complicated by the fact that CO_2 and H_2O are always in rapid equilibrium with H_2CO_3, according to the reaction

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \quad (5)$$

While CO_2 dissolved in H_2O will reach chemical equilibrium very rapidly ($t_{1/2} \approx$ 19 s; Gibbons and Edsall 1963), isotopic equilibrium between all the oxygens takes about 1000 times longer (Mills and Urey 1940). This is because for isotopic equilibrium every molecule must come to equilibrium with every other molecule; therefore, the hydration reaction must occur many times. Chemical equilibrium, on the other hand, is dependent only on the ratio of forward to reverse rate constants; as soon as $[H_2CO_3]$ reaches its equilibrium value, the forward and reverse rates are equal, and chemical equilibrium is achieved. The time required to reach isotopic equilibrium is very sensitive to the pH, since the greater the ratio of $[HCO_3^-]/[CO_2]$ (i.e. the higher the pH), the fewer the number of hydration reactions in a given time. Because of the very slow oxygen exchange at the temperature (25 °C) and pH ($\sim$ 10) of the ^{18}O experiments with *Chlorella*, the original investigators felt justified in ignoring this complication, as the oxygen exchange was much slower than the rate of photosynthesis. However, Kamen and Barker (1945) pointed out that it was an unproven assumption that the isotope exchange is no more rapid inside the cells, or inside the chloroplasts, than in the outside medium. Assuming an internal pH of 6 or less, these authors calculated that the randomization of ^{18}O would be rapid enough to invalidate the conclusions.

Because of its heavier mass, CO_2 is slightly more enriched in ^{18}O than is H_2O at isotopic equilibrium (Webster et al. 1935). Dole and Jenks (1944) were able to sensitively measure this difference, and they showed that in photosynthesizing cells in which isotopic equilibrium was maintained with carbonic anhydrase, the evolved O_2 had nearly the same enrichment as the H_2O, but was clearly less enriched than the CO_2, thus confirming the original conclusions. Since this experiment was done at isotopic equilibrium, it was not subject to the same criticism as the first experiments.

This line of evidence still had difficulties. It has long been presumed that the source of the earth's atmospheric O_2 is photosynthesis, yet the ^{18}O content of atmospheric O_2 is considerably greater than that of natural water (Dole 1935). Green and Voskuyl (1936) pointed out that the ^{18}O content of atmospheric O_2 is what would be predicted if photosynthetic O_2 were derived from the water and CO_2 together. Yosida et al. (1942) claimed to have measured an ^{18}O content of photosynthetic O_2 evolved from aqueous plants that indicated one-third of the O_2 came from CO_2. They were able to account for this observation using a modified Willstätter-Stoll hypothesis for O_2 evolution, in which carbonic acid (i.e. hydrated CO_2) yields formaldehyde and O_2 through a peroxide intermediate. The experiment of Dole and Jenks

(1944), described above, contradicts these results and shows clearly that, at least in *Chlorella*, the O_2 evolved is much closer in isotopic composition to the H_2O than it is to the CO_2. Still, they did show a slight increase in ^{18}O abundance compared to the H_2O; this they noted is precisely what would be predicted if there were an efficient oxygen exchange between O_2 and H_2O, although from the earlier measurements of Ruben et al. (1941) it was concluded that such an exchange did not occur. Given the complications of oxygen exchange reactions, the greater ^{18}O content of atmospheric O_2 compared to natural H_2O, and the small number of species tested, these ^{18}O labeling experiments have not escaped controversy. We refer the reader to Rabinowitch (1945, 1951, 1956) for further references and details. In our opinion the original experiments of Ruben et al. (1941) and of Dole and Jenks (1944) were carefully done. However, we note that Warburg (1964) and Metzner (1975) have challenged their validity. Thus, further research is still necessary.

Stemler and Radmer (1975) measured the isotopic O_2 released from HCO_3^--depleted thylakoids after the addition of $HC^{18}O_3^-$ and found that only $^{16}O_2$ was evolved. This experiment was superior to the original experiments of Ruben et al. (1941) in that the appearance of $C^{18}O_2$ and $C^{16}O_2$ were monitered along with the evolution of $^{16}O_2$, and it was shown that the isotopic exchange reactions were too slow for HCO_3^- or CO_2 to be the source of photosynthetic O_2. Also, since the thylakoids had been depleted of native $HC^{16}O_3^-$, the $^{16}O_2$ could only have come from $H_2{}^{16}O$. However, since the mass spectrometer used in this experiment was not sensitive enough to moniter single turnovers of the reaction centers, these authors could not rule out the remote possibility that the first few molecules of evolved O_2 might have been derived from the reactivating HCO_3^-. This qestion was apparently settled by Radmer and Ollinger (1980) who showed that even after an actinic flash, the isotopic composition of the evolved O_2 matches that of the H_2O and not of the HCO_3^-. It would seem, then, that there is little reason to doubt that H_2O is the substrate for O_2 evolution. However, the validity of even these experiments has been questioned by Stemler (1982), who holds open the possibility that PS II may be able to catalyze the hydration of CO_2 (and therefore the isotopic exchange reactions) at the O_2 evolving site.

The third major line of evidence that H_2O is the substrate for photosynthetic O_2 evolution was the classical observation by Hill (1937, 1939) that broken chloroplasts could be made to evolve O_2 in the light by the addition of ferric oxalate. This was the first successful observation of photosynthetic O_2 evolution in a system other than whole cells or an intact leaf. It was not necessary for Hill to supply the chloroplasts with CO_2; in fact, he made his observation with the chloroplasts evacuated, so any CO_2 remaining was very

low in concentration. At first it was thought that some O_2 had to be present, but Hill and Scarisbrick (1940a) later found that this was simply because the small amount of ferric oxalate added was quickly reduced by organic acids present, and the O_2 was necessary to reoxidize the ferrous oxalate to ferric oxalate. If no exhaustion of the ferric salt was allowed to occur, the evolution of O_2 could continue for several hours. From the behavior of the reaction with various inhibitors, Hill and Scarisbrick (1940b) concluded that the O_2 was evolved in the "light" reaction, and that CO_2 was reduced in dark reactions that were disrupted during chloroplast isolation. The ferric salts stimulated O_2 evolution by acting as an oxidant (electron acceptor) for the "light" reactions. This physical separation of the reactions leading to O_2 evolution from the reactions catalyzing CO_2 fixation was strong evidence for the non-involvement of CO_2 in O_2 evolution. Furthermore, the observed reduction of the added electron acceptor (now referred to as a "Hill oxidant"; electron transport in isolated thylakoids was called the "Hill reaction", we presume, by French et al., (1946)) confirmed that the "light" reactions of photosynthesis were oxidation-reduction reactions, with H_2O being the terminal reductant. Thus, the evolution of O_2 could be seen as a simple oxidation:

$$2\ H_2O \rightarrow O_2 + 4\ H^+ + 4\ e^- \tag{6}$$

To sum up the discussion so far, three major lines of evidence have contributed to the current picture of H_2O as the source of photosynthetic O_2 evolution: (i) by comparison with photosynthetic bacteria, which use light energy to reduce CO_2 and oxidize a hydrogen donor, plant photosynthesis is presumed to operate by a similar mechanism, with H_2O as the hydrogen donor; (ii) ^{18}O labeling experiments have shown that the O_2 that is evolved is labeled to the same extent, or to nearly the same extent, as the H_2O, but not as the CO_2; and (iii) the "light" reactions of photosynthesis can be separated from the dark reactions; O_2 is evolved in the "light" reactions, while CO_2 is reduced in the dark reactions. The first two lines of evidence have difficulties inherent in the method that make them less than conclusive: (i) photosynthetic bacteria have major fundamental differences from photosynthetic plants, such as in the gross architecture, in the number of photosystems, etc., and it has been argued that a direct comparison is not necessarily valid; and (ii) the complications of natural oxygen exchange between CO_2 and H_2O have cast doubt on the validity of the ^{18}O experiments in the minds of some researchers.

Given these objections to the first two lines of evidence, the third line of evidence, the independence of the Hill reaction on CO_2, became a corner-

stone argument for H_2O as the source of photosynthetic O_2. It was therefore of great importance when Warburg and Krippahl (1960) showed conclusively that the Hill reaction was reversibly inhibited by removal of CO_2 from the membranes. Although the Hill reaction did not require that CO_2 be supplied to the membranes, Hill did not actually remove CO_2 that might have already been bound to the membranes. When this CO_2 is removed, the Hill reaction ceases. The implication is that if O_2 evolution cannot occur in the absence of CO_2, then CO_2 could remain a candidate for the source of evolved O_2. Even if H_2O is also shown to be involved, this does not preclude hydrated CO_2 (i.e. H_2CO_3 or HCO_3^-) as the source, as originally proposed by Willstätter and Stoll. This hypothesis, and variations of it, have been proposed throughout the nearly three decades since Warburg and Krippahl's discovery (e.g. see Warburg 1964, Stemler 1982).

Despite the above objections, the scientific community has not seriously questioned H_2O as the source of evolved O_2 since the time of Hill and Bendall's very successful "Z-scheme" (Hill and Bendall 1960; for reviews, see Govindjee 1975, 1982). The accumulated evidence for the light reactions of photosynthesis being a series of oxidation-reductions, with H_2O as the ultimate source of electrons, is overwhelming, and the picture is not likely to be overturned easily. It is possible, of course, to incorporate CO_2 into the mechanism of O_2 evolution without a major overhaul of our current model – the mechanism of O_2 evolution is still one of the least understood aspects of photosynthesis (see reviews by Renger and Govindjee 1985, Govindjee et al. 1985, Babcock 1987, Renger 1987) – but models such as that championed by Warburg (1964), in which CO_2 has more than a regulatory or catalytic role, are probably out of vogue forever. One effect of the controversy over the past 25 years seems to have been not so much to cast doubt on the role of H_2O, as to have raised suspicion or caused indifference to the role of HCO_3^-. This is unfortunate, because although a major effect of HCO_3^- on O_2 evolution can be ruled out, it very clearly does play a major role in the reduction of plastoquinone (see reviews by Govindjee and van Rensen 1978, Vermaas and Govindjee 1981a, 1982a, van Rensen and Snel 1985, Govindjee and Eaton-Rye 1986) and is, therefore, deserving of more attention than it has received. This paper is one attempt to redress this imbalance. We first examine below what is known about the role of HCO_3^- in PSII.

The requirement for HCO_3^- in PS II

The discovery

Credit for the discovery of the HCO_3^- requirement in the light reactions of

photosynthesis is generally given to Warburg and Krippahl (1958, 1960). However, such a requirement was suggested several years earlier by Boyle (1948), who quite by accident noticed that O_2 production was halted in the H_2O to p-benzoquinone Hill reaction in ground spinach leaves when KOH was placed in the center well of a manometer vessel to take up CO_2. This observation was shown later to be an artifact (Abeles et al. 1961), due to distillation of quinone from the main compartment of the reaction vessel into the KOH solution. Under such conditions, O_2 uptake occurs in the center well at a rate sufficient to reabsorb all of the O_2 produced by the Hill reaction. Thus, no net O_2 evolution would have been observed by Boyle, regardless of whether CO_2 were required or not. The conclusion that CO_2 was required was, therefore, unwarranted. Under the same conditions as Boyle, Abeles et al. (1961) found no effect on the Hill reaction by having CO_2 present or absent in the vessel. On the other hand, the same authors were able to confirm the observation by Warburg and Krippahl, noting, under their conditions, a consistent difference in O_2 evolution when atmospheric CO_2 was present or absent. In these experiments the quinone concentration was lower, and there was no KOH in the center well. This, along with differences in pH, probably accounts for the failure to observe a CO_2 dependence under Boyle's conditions.

Other researchers may have noticed the requirement for HCO_3^- earlier, without fully recognizing it as such. For instance, Franck (1945) reported that broken chloroplasts evolved more O_2 when they were supplied with CO_2 than when they were flushed with N_2. Admission of CO_2 to CO_2-free suspensions always caused a sudden increase in the rate of O_2 production. Franck took this observation to justify that he was looking at real photosynthesis, with CO_2 as oxidant. However, from his protocol it is clear that he was using broken chloroplasts, which would have been missing the stromal enzymes necessary for CO_2 reduction. He did not add a Hill oxidant to his preparations; only small amounts of O_2 were evolved, but the rate was significantly higher when CO_2 was present. Later, under the same experimental conditions, Brown and Franck (1948) found that when $^{14}CO_2$ was used, there was no accumulation of the radiolabel in the chloroplasts. Therefore, they concluded that the stimulation by CO_2 was not due to CO_2 fixation, but to some other, possibly catalytic, role for CO_2.

This observation did not attract much attention until Warburg and Krippahl (1958, 1960) rediscovered the requirement for CO_2. They, too, showed that the Hill reaction was inhibited by CO_2 removal and strongly stimulated by addition of CO_2 at low partial pressure, and that there was no net reduction of CO_2 occurring simultaneously. These authors suggested that the CO_2 requirement reflects a catalytic function for CO_2 in the mechan-

ism of O_2 evolution. Earlier, Burk and Warburg (1950) had postulated an elaborate scheme for photosynthesis which contradicted much of the collective wisdom of the photosynthesis community, and this new finding of a CO_2 requirement was quickly pounced upon as evidence for the scheme (Warburg et al. 1959). In Warburg's scheme, which did not separate the photochemical process from CO_2 metabolism, a photochemical reaction consumes one molecule of CO_2 and yields one molecule of O_2 per quantum, then a thermochemical back reaction consumes two-thirds of the released O_2 and releases two-thirds of the consumed CO_2. To explain the Hill reaction, in which no net reduction of CO_2 occurs (Brown and Franck 1948, Warburg and Krippahl 1958), Warburg and Krippahl postulated that unlike whole cells, isolated chloroplasts cannot retain the reduced CO_2, which is reoxidized by the Hill reagent. In Warburg and Krippahl's scheme the precursor of O_2 is a phosphorylated peroxide of carbonic acid, produced by the action of illuminated chlorophyll on the Hill oxidant, CO_2, H_2O, and phosphate.

Perhaps because of Warburg's insistence that H_2O is not the source of photosynthetic O_2, this observation of a CO_2 requirement, unlike the earlier observation of Franck (1945), attracted much notice, and was rapidly confirmed by several researchers (Abeles et al. 1961, Stern and Vennesland 1962, Izawa 1962, Good 1963). The CO_2 effect was shown to be a general phenomenon, observable with a wide variety of Hill reagents and with a wide variety of species (Stern and Vennesland 1962). Several observations, especially by Norman Good, at this time argued against the scheme of Warburg and Krippahl: (i) the stimulatory effect of CO_2 on the Hill reaction was much reduced in weak light, compared to strong light, suggesting that CO_2 was not involved in a photochemical reaction, but in a non-photochemical step (Izawa 1962, Good 1963); (ii) the correlation of the CO_2 dependence with the presence of small anions suggested that HCO_3^-, not CO_2, was the important substance (Good, 1963); (iii) whereas Warburg's scheme has photophosphorylation intimately connected with CO_2 metabolism, uncouplers of phosphorylation do not relieve the impairment caused by CO_2 depletion, indicating a site of action remote from phosphorylation (Good 1963); and (iv) whereas one would expect a greater CO_2 dependence with weaker Hill oxidants if the oxidant is involved in CO_2 metabolism, no such trend was observed (Good 1963). Nevertheless, Warburg continued to present his scheme as though it were established fact (c.f. Warburg 1964).

The site of action

Because of the non-independence of the HCO_3^- effect on light intensity, it was concluded that HCO_3^- acts at a non-photochemical step of the Hill

reaction (Izawa 1962, Good 1963). The first attempt to locate this site of action was by Punnett and Iyer (1964), who looked at the effect of CO_2 on photophosphorylation. They observed that by adding relatively high concentrations of HCO_3^- to non-HCO_3^--depleted chloroplasts, they could accelerate the Hill reaction, as well as enhance the rate of phosphorylation. The ATP:$2e^-$ ratio was also increased, particularly when the pH was above 7. Thus, one of the effects of added CO_2 appeared to be to improve the coupling between electron transport and phosphorylation. However, as pointed out by Batra and Jagendorf (1965), the stimulation of the Hill reaction by high [HCO_3^-] in the absence of either uncouplers or ADP and phosphate seems to argue, if anything, for a looser coupling. The apparent contradiction of these two observations they found difficult to rationalize. Punnett and Iyer suggested that CO_2 may increase the efficiency of formation of a high energy intermediate resulting from electron transport (now understood to be a pH difference across the membrane), but Batra and Jagendorf found that added CO_2 actually decreases the yield of the high energy state of the chloroplasts, which suggested to them that the high energy state may be in competition with the formation of ATP (it is now understood that ATP synthesis occurs at the expense of the transmembrane pH difference).

Batra and Jagendorf extended the observations of Punnett and Iyer and showed that the effect observed by them is actually a different effect than the HCO_3^- dependence observed by Franck (1945) and by Warburg and Krippahl (1958, 1960): (i) the *Punnett and Iyer effect* requires a relatively high [HCO_3^-] added to non-HCO_3^--depleted chloroplasts, whereas the *Franck/Warburg effect* requires much lower concentrations of HCO_3^- added to HCO_3^--depleted chloroplasts; (ii) uncouplers eliminate the stimulation of the Hill reaction by HCO_3^- in non-depleted chloroplasts (Batra and Jagendorf 1965), whereas uncouplers have no effect on the HCO_3^- dependence of depleted chloroplasts (Stern and Vennesland 1962, Good 1963, Khanna et al. 1977); (iii) added HCO_3^- stimulates phosphorylation under conditions of cyclic electron flow around PS I, supported by pyocyanine, with or without CMU (p-chlorophenyl-1,1-dimethyl urea) to block electron donation by PS II, whereas the removal of CO_2 by depletion has no effect on pyocyanine supported phosphorylation (Batra and Jagendorf 1965); and (iv) the *Franck/Warburg effect* appears to represent a requirement for HCO_3^-, in that the rate of electron transport is depressed by removal of CO_2 and is restored by adding back the HCO_3^-, whereas the *Punnett and Iyer effect* is a true stimulation, in that removal of CO_2 does not inhibit phosphorylation and cyclic electron transport (Batra and Jagendorf 1965). To this list can be added several other observations: the pH optimum for the *Franck/Warbuck effect* is around pH 6.5 (Khanna et al. 1977, Vermaas and van Rensen 1981),

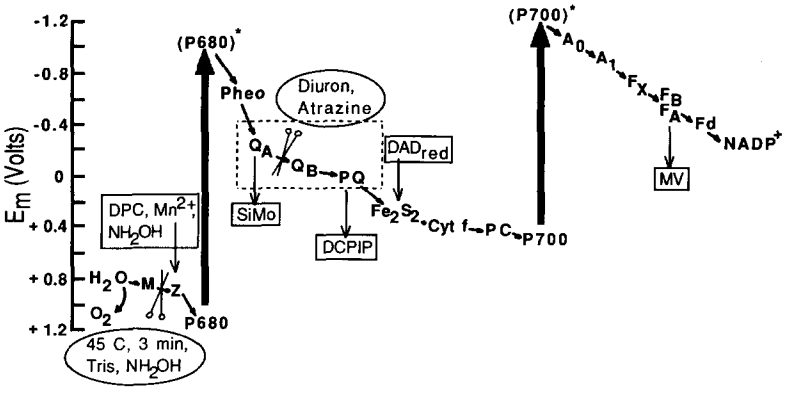

Fig. 1. Sites of electron donors, acceptors and inhibitors in the noncyclic electron transfer pathway of plant photosynthesis. The dashed box encloses the steps that are inhibited by removal of HCO_3^-. The solid boxes enclose exogenous redox compounds which act as artificial electron donors or acceptors at the step indicated by the arrows. DPC is diphenylcarbazide; SiMo is silicomolybdate; DCPIP is 2,6-dichlorophenolindophenol; DAD_{red} is reduced diaminodurene; MV is methylviologen. The arrows point in the direction of electron flow. The ellipses enclose treatments or compounds which interrupt electron flow at the step indicated by the scissors. The photosynthetic components are placed vertically according to their approximate midpoint redox potential (E_m). The exogenous compounds enclosed by solid boxes or ellipses are placed for diagramatic convenience, not according to their redox potentials. Electron flow is initiated with a photon or an exciton at the reaction center chlorophyll *a* molecules of photosystem II (P_{680}) or photosystem I (P_{700}). The asterisks indicate excited states. M is the charge accumulator and the oxygen evolving complex; Z, thought to be tyrosine-160 of the D_1 reaction center polypeptide, is the electon donor to P_{680}; Pheo represents pheophytin; Q_A is a plastoquinone (PQ) molecule permanently bound on the D_2 polypeptide; Q_B is a PQ molecule transiently bound on the D_1 polypeptide -- after reduction to plastoquinol, Q_B (H_2) exchanges with another PQ molecule; PQ represents a pool of non-bound plastoquinone; Fe_2S_2 represents the Rieske iron-sulphur center; Cyt f stands for cytochrome f; PC is a plastocyanin; A_o is suggested to be a chlorophyll molecule; A_1 is possibly a quinone, phylloquinone; F_A, F_B and F_X are thought to be Fe-S centers; Fd is soluble ferredoxin; $NADP^+$ is nicotinamide adenine dinucleotide phosphate (also see Govindjee and Eaton-Rye 1986).

whereas Punnett and Iyer observed the maximal effect between pH 7.0 and 7.8; the *Franck/Warburg effect* requires a dark incubation for HCO_3^- to bind (Stemler and Govindjee 1973, Vermaas and van Rensen 1981), whereas the binding of HCO_3^- to a low affinity site occurs preferentially in the light (Blubaugh and Govindjee 1984); and herbicide binding appears to overlay HCO_3^- added to depleted thylakoids, but not HCO_3^- binding to a low affinity site in non-depleted thylakoids (Blubaugh and Govindjee 1984).

The first attempt to locate the site of impairment in HCO_3^- depleted chloroplasts (*Franck/Warburg effect*) was the study by Stemler and Govindjee (1973), which seemed to show that HCO_3^- depletion had no effect on the rate of electron transport from the artificial PS II electron donor diphenylcarbazide (DPC) to the electron acceptor 2,6-dichlorophenolindophenol

(DCPIP) (Fig. 1). DPC is believed to donate electrons to the primary electron donor to the reaction center of PS II, Z. Therefore it was concluded then that the effect of HCO_3^- depletion on the H_2O to DCPIP reaction was due to a HCO_3^- site prior to Z; that is, at the O_2 evolving locus itself. However, this conclusion was soon abandoned as the result was reinterpreted to be due to the rate-limiting donation of electrons by DPC, which obscures the HCO_3^- effect (Wydrzynski and Govindjee 1975). This scenario was repeated a decade later when Fischer and Metzner (1981) concluded that HCO_3^- was required at the O_2 evolving site, in part because they could not observe a HCO_3^- effect in thylakoids using artificial electron donors to PS II (hydroxylamine, Mn^{2+}, a tetramethylbenzidine and tetraphenylboron). Eaton-Rye and Govindjee (1984) showed that for at least two of these (hydroxylamine and benzidine), the electron transport rates supported by these donors is no greater in non-depleted controls than the rates typically obtained by HCO_3^- depletion; thus, this approach cannot be used to assign a location for the HCO_3^- impairment.

Another problem with DPC as an electron donor is that it has an apparent effect on the membrane structure; in thylakoids that have begun to break down, DPC appears to stimulate energy trapping by PS II, perhaps by linking physically separated components (Harnischfeger 1974). This increase in the quantum yield can mask an impairment of electron transport. However, the HCO_3^- effect can be seen with DPC and other artificial electron donors to PS II, if one looks at chlorophyll *a* fluorescence instead of electron transport (for recent reviews on chlorophyll *a* fluorescence, see Govindjee et al. 1986). Wydrzynski and Govindjee (1975) showed that HCO_3^- depletion accelerates the rise of the chlorophyll *a* fluorescence transient in a manner similar to the herbicide diuron (3-(3,4-dichlorophenyl)-1, 1-dimethylurea, DCMU), which is known to "block" electron transport after the first stable plastoquinone acceptor, Q_A (Fig. 1). In contrast, treatments which are known to impair the O_2 evolving mechanism, such as mild heat treatment, Tris treatment, etc., were shown to eliminate the variable chlorophyll *a* fluorescence. These effects are predictable from the understanding that chlorophyll *a* fluorescence is a moniter of $[Q_A^-]$ (Duysens and Sweers 1963, Murata et al. 1966). Since HCO_3^- depletion produces a transient similar to treatment with diuron, Wydrzynski and Govindjee were the first to conclude that HCO_3^- depletion causes a block on the acceptor side of PSII, after Q_A. In support of this argument, they showed that DPC, as well as other artificial PS II donors, restore the variable fluorescence to heat-treated and Tris-treated chlororplasts, but the effects of HCO_3^- depletion and restoration remain, even with these donor systems. Similarly, Eaton-Rye and Govindjee (1984) showed that when hydroxylamine is used

to simultaneously inhibit O_2 evolution and to donate electrons to PSII, the decay of chlorophyll *a* fluorescence after a flash, which moniters the reoxidation of Q_A^-, is reversibly inhibited by HCO_3^- depletion. Thus, they reaffirmed the location of the HCO_3^- requirement to be on the acceptor side of PS II, after Q_A.

Initially, Stemler and Govindjee (1974) had interpreted the effect of HCO_3^- depletion on the chlorophyll *a* fluorescence transient as supportive of an impairment on the O_2 evolving side of PS II. This is because, although they observed an acceleration of the rise from the initial level F_O to the intermediate hump I (F_I), they had observed a slower rise from F_I to the maximum fluorescence level F_{max} (F_P). They had argued that a block after Q_A would have caused a higher fluorescence at all times. However, this transient can now be better understood as a partial block after Q_A, due to a partial HCO_3^- depletion in their experiments. The accelerated rise from F_O to F_I is due to the faster accumulation of Q_A^-, while the slower rise from F_I to F_{max} represents the filling of the plastoquinone (PQ) pool, which is slowed by the impairment; only when the PQ pool is reduced can $[Q_A^-]$ accumulate to its maximum level (Vermaas and Govindjee 1981a). A thorough HCO_3^- depletion causes a complete, or nearly complete, block between Q_B and the PQ pool, causing a fluorescence transient which is indeed higher at all times, up to F_{max} (Vermaas and Govindjee 1982b).

It is now well established that the major site of impairment caused by HCO_3^- depletion is on the electron acceptor side of PS II (for a review of this side, see Vermaas and Govindjee 1981b). By the use of artificial electron donors and acceptors, Khanna et al. (1977) suggested that the site was between Q_A and PQ. The PS I electron transport, as measured by O_2 uptake during electron transport from reduced diaminodurene (DAD_{red}) to methylviologen (MV) (DAD_{red} donates electrons after the PQ pool; MV accepts electrons from the terminal side of PS I and passes them to O_2; see Fig. 1), did not show any bicarbonate effect. Since the rates of electron flow were very high indeed, it is firmly established that HCO_3^- is not involved in these reactions. The PS II electron transport prior to Q_A, as measured by O_2 evolution during electron transport from H_2O to silicomolybdate (SiMo), also remained uninhibited by HCO_3^- depletion. However, the PS II reduction of oxidized DAD, which efficiently accepts electrons from the PQ pool, did show a strong HCO_3^- dependence. Although the SiMo result cannot be taken to prove the absence of the HCO_3^- effect in this reaction, again, due to the low rates of electron flow, all the results (see later) taken together suggest that the site of inhibition is after Q_A, but before the PQ pool. Graan (1986) has challenged the generally accepted premise that SiMo accepts electrons from Q_A (e.g. Giaquinta and Dilley 1975, Zilinskas and Govindjee

1975). He argues that all available evidence concerning SiMo involvement with PS II is also consistent with SiMo simply replacing diuron from the Q_B binding site (see also Böger 1982). The apparent ability to replace diuron is dependent on the redox state of SiMo; the reduced form apparently binds not all all or much less tightly than the oxidized form (Graan 1986). Thus, SiMo may be functioning like benzoquinone and other electron acceptors which replace PQ, except that the binding affinity of the oxidized form is high enough to outcompete diuron. Therefore, many reported observations throughout the literature, including the absence of a HCO_3^- effect in the H_2O-to-SiMo reaction, may have to be re-evaluated if Graan is correct. However, if the reduction of SiMo is rate limiting, then an impairment of electron transport after Q_A by HCO_3^- depletion would not be expected to be seen.

Regardless of what the final outcome concerning SiMo will be, there remains ample evidence for the involvement of HCO_3^- in electron transport between Q_A and the PQ pool. Jursinic et al. (1976) were the first to demonstrate that HCO_3^- depletion slowed the oxidation of Q_A^-, and, consequently reduction of the next electron acceptor Q_B, as monitered by the decay of chlorophyll *a* fluorescence yield after an actinic flash, from a half-time of about 0.5 ms to approximately 2.6 ms. When the decay was determined as a function of flash number (Govindjee et al. 1976), it was discovered that the oxidation of Q_A^- was even slower after the third and subsequent flashes, with a half-time of about 150 ms. Since Q_B acts as a "two electron gate" (see e.g. review by Vermaas and Govindjee 1981b), this suggests that two electrons can still flow through Q_A to reduce Q_B to Q_B^{2-}, and that the reoxidation of Q_B^{2-} then becomes rate limiting. Thus, HCO_3^- depletion appears to slow down electron flow from Q_A to Q_B and to block the exchange of Q_B^{2-} with the PQ pool.

There also appeared to be a 30-50% inhibition of charge separation in these and other repetitive flash experiments (Stemler et al. 1974, Jursinic et al. 1976, Siggel et al. 1977), which prompted the suggestion that HCO_3^- depletion also inactivates a portion of the PS II reaction centers (Jursinic et al. 1976). However, an alternative explanation was offered by Jursinic and Stemler (1982), who found that a very slow component of the fluorescence decay, with a half-time of 1-2 s, was increased two-to-three-fold in HCO_3^- depleted samples. They suggested that in a significant portion of the reaction centers of HCO_3^- depleted chloroplasts, Q_A^- was not reoxidized in the dark time between flashes, thus keeping the reaction centers in a photosynthetically closed state. Since the increase of this very slow component occurred even after the first flash, they concluded that it was a component of the Q_A^- to Q_B electron transfer, and they suggested that HCO_3^- depletion may alter

the redox potential (Em, 7) of Q_A with respect to Q_B, or reduce a local field that stabilizes Q_B^-. Vermaas and Govindjee (1982b) did not find any effect of HCO_3^- on the redox potential of Q_A/Q_A^-. It has been suggested that HCO_3^- depletion does destabilize Q_B^- by preventing the protonation of a nearby protein group (Eaton-Rye 1987). It is also possible that this slow component represents some inactive PS II centers (e.g. Graan and Ort 1986, Garab et al. 1987), and that HCO_3^- depletion somehow raises the number of such centers, perhaps by inhibiting the binding of PQ (e.g. Eaton-Rye 1987, see also Blubaugh 1987). It is possible that these slow centers are inactive they don't have HCO_3^- bound to them, an idea that is also shared by J. Whitmarsh, (personal communication). In normal active centers, PQ binding and release must occur with a half-time less than 1 ms (Crofts et al. 1984), in order to account for the observed reduction time of the PQ pool (Stiehl and Witt 1969).

Robinson et al. (1984) confirmed the slower chlorophyll fluorescence decay of HCO_3^- depleted thylakoids, but obtained much faster rates, overall, than were reported by Govindjee et al. (1976). Presumably, this was due to a slower flash frequency (1 Hz, instead of 33 Hz) that permitted most of the very slow component to decay between flashes. After one or two flashes, Q_A^- decays with a half-time of 1.2 ms in HCO_3^- depleted thylakoids, compared to 0.23 ms in the control samples. After 3 flashes the half-time is increased to 10 ms. Eaton-Rye (1987) has extended these observations to show that at pH 7.5, the half-time of Q_A^- decay in HCO_3^- depleted thylakoids continues to increase after each flash up to 5–7 flashes, as opposed to only 3 flashes at pH 6.5. Also, after one or two flashes, the half-time is greater at the acidic pH, whereas after 4 or more flashes the half-time is greater at the alkaline pH. This behavior was explained by two separate pH-dependent processes. From a kinetic analysis of the pH dependence of the decay rate after 1–2 flashes, it was suggested that the binding of PQ is inhibited by HCO_3^- depletion, with the greatest effect occurring at acidic pH. A simultaneous inhibition of the protonation of Q_B^- was presumed to occur, which becomes more severe at alkaline pH because of the further reduced availability of H^+.

The inhibition of Q_A^- reoxidation by HCO_3^- depletion has also been shown by following the decay of the absorbance change at 320 nm, which is due to absorption by the semiquinones Q_A^- and Q_B^-, with comparable results to those obtained by the fluorescence decay experiments described above (Siggel et al. 1977, Farineau and Mathis 1983).

The site of HCO_3^- action has also been located at the quinone reactions by the interaction between HCO_3^- binding and herbicide binding. Khanna et al. (1981) showed that HCO_3^- depletion decreased the binding affinity of atrazine. Similarly, a variety of atrazine-type herbicides have been shown to inhibit HCO_3^- binding (van Rensen and Vermaas 1981, Vermaas et al. 1982,

Snel and van Rensen 1983). Most of these herbicides appear not to be competitive with HCO_3^-, but bind closely enough to be affected by it. Since these herbicides are believed to inhibit PS II by replacing PQ from the Q_B site (e.g. Oettmeier and Soll 1983), the binding of HCO_3^- at or near Q_B is presumed.

While the effect of HCO_3^- depletion on the acceptor side of PS II has been firmly established, an effect on the donor side has been a source of controversy. Numerous observations have been reported to support the idea of a major effect on the O_2 evolving complex (for a review, see Stemler 1982). However, most of these observations have been explained without the need to invoke a significant site on the donor side of PS II (e.g. Vermaas and Govindjee 1981a, 1981b, 1982a). A small effect is, however, well known in which HCO_3^- can replace Cl^- to some extent on the electron donor side of PS II (see e.g. Critchley et al. 1982, Jursinic and Stemler 1988). On the other hand, some of the effects on the S-state transitions of the O_2 evolving complex (Stemler et al. 1974, Stemler 1980) may be due to interactions of the S-states with the Q_B site (see e.g. Diner 1977, Govindjee et al. 1984). Some of the observations have been shown to be artifactual, such as the apparent insensitivity of electron transport supported by artificial PS II donors (discussed above); and an effect on the kinetics of O_2 evolution after a flash could not be confirmed (Vermaas and Gonvindjee 1982a). One observation, that the rate of $H^{14}CO_3^-$ binding appears to be dependent on the pH of the lumen, rather than the external pH (Stemler 1980), has an alternative interpretation (see *The Model*). No firm evidence has, to date, been shown for a significant involvement of HCO_3^- on the donor side of PS II. In fact, numerous studies suggest a non-involvement of HCO_3^- on the donor side (Stemler and Radmer 1975, Jursinic et al. 1976, Khanna et al. 1977, van Rensen and Vermaas 1981, Khanna et al. 1981); this, of course, does not contradict the inefficient and small effect of HCO_3^-, like other monovalent anions, in replacing Cl^- on the donor side of PS II.

The active species

As was discussed in previous sections, the requirement for CO_2 in the Hill reaction was originally thought to indicate an involvement of CO_2 in the O_2 evolving mechanism (Warburg et al. 1959, see also Stemler and Govindjee 1973). Warburg and coworkers (1959) developed an elaborate scheme to show how a phosphorylated peroxide of carbonic acid (hydrated CO_2) could be the precursor to O_2 evolution. This scheme assumed that CO_2 or H_2CO_3 was the species required.

Good (1963) was the first to examine the effect of various anions on the

bicarbonate (HCO_3^-) dependence, and found that small monovalent anions increased the dependence of the chloroplasts on HCO_3^-. Particularly effective were formate (HCO_2^-) and acetate ($CH_3CO_2^-$), which suggested to Good that the HCO_3^- ion is the important substance, not CO_2. Stemler and Govindjee (1973) took advantage of this suggestion to obtain the first method for reproducibly obtaining what was then considered to be a large (4–5 fold) HCO_3^- dependence of ferricyanide or 2,6-dichlorophenol indophenol (DCPIP) reduction by isolated chloroplasts. Their treatment consisted of low pH to favor the conversion of HCO_3^- to CO_2 and high salt (250 mM NaCl, 40 mM Na acetate) to compete with HCO_3^- for its binding site. Their maximum restored rates, however, were still largely inhibited with respect to non-treated chloroplasts, an effect which today can be understood as a consequence of irreversible damage by low pH (see e.g. Vermaas and Govindjee 1982b).

A subsaturating [HCO_3^-] showed a larger stimulation of the Hill reaction at pH6.8 than it did at pH 5.8, supporting the suggestion that HCO_3^- is the active species (Stemler and Govindjee 1973). This experiment was not conclusive, however, since it did not rule out the possibility of a pH dependence on the binding affinity of the active site. The authors favored the conclusion that HCO_3^- is the active species, however, by another argument: They showed that HCO_3^- stimulation of the Hill reaction only occurred when the chloroplasts were incubated with HCO_3^- in the dark. No stimulation occurred while the chloroplasts were illuminated, though a subsequent dark period would restore the activity. It was suggested that HCO_3^- is released in the light at a rate that corresponds with its binding. Since CO_2 is uncharged and non-polar, it would not be expected to bind other than by covalent attachment, while the suggested exchange is more consistent with an ionic binding. Thus, HCO_3^- as the active species was thought to be more likely. However, CO_2 can form relatively unstable carbamate complexes with protein amino groups, which decompose readily. Such a carbamate formation, for instance, has been demonstrated for the regulation of ribulose-1,5-bisphosphate carboxylase by CO_2 (Lorimer et al. 1976).

The pH profile of the HCO_3^- dependence shows an optimum around pH 6.5 (Khanna et al. 1977, Vermaas and van Rensen 1981). While the measurements of Stemler and Govindjee (1973) were confirmed by the pH profile, the drop-off of the dependence as the pH is increased above 6.5 argued against HCO_3^- as the sole active species, since [HCO_3^-] would be expected to increase with increasing pH up to around pH 8.4. Again, this argument ignores any possible pH effects on the binding environment. However, because of the close proximity of the pH optimum to the pKa of HCO_3^-/CO_2

(pKa = 6.4), it was suggested that both CO_2 and HCO_3^- are required (Vermaas and van Rensen 1981).

Evidence that CO_2 is involved in the "bicarbonate effect" was obtained by Sarojini and Govindjee (1981a, 1981b) by measuring the lag time between the addition of CO_2 or HCO_3^- and the onset of O_2 evolution. At a low assay temperature (5 °C) to slow the interconversion of CO_2 and HCO_3^-, and an alkaline pH of 7.3 to give a large ratio of $[HCO_3^-]$ to $[CO_2]$, the lag time was considerably shorter when CO_2 was added, compared to HCO_3^-. When cabonic anhydrase was present to accelerate the interconversion of CO_2 and HCO_3^-, the lag times became nearly identical for either species added. These results were interpreted to mean that either CO_2 was the species that was bound, or that CO_2 was required for diffusion to the active site.

Stemler (1980) concluded that CO_2, not HCO_3^-, is the binding species because in non-depleted chloroplasts inhibited by 100 mM HCO_2^-, the addition of 50 mM HCO_3^- caused a further inhibition of O_2 evolution at pH 8.0, whereas HCO_3^- partially restored the activity at pH 7.3. Because of the much larger $[CO_2]$ at the lower pH, it was concluded that CO_2 is the binding species and that HCO_3^-, like HCO_2^-, is inhibitory. However, these measurements, like the earlier ones at pH 5.8 and 6.8 (Stemler and Govindjee 1973), are not sufficient to determine the active species, since they ignore possible pH effects on the binding environment.

Stemler (1980) observed that the rate of ^{14}C labeling of HCO_3^- depleted chloroplasts by $H^{14}CO_3^-$ decreases with increasing pH over the pH range of 6.0 to 7.8, provided that at least a 5 min incubation period is given at the pH before the $H^{14}CO_3^-$ is added. When the incubation was omitted, the rate of ^{14}C labeling was pH independent for at least two minutes. These observations led Stemler to conclude that not only is CO_2 the binding species, but that the binding ocurs on the inside surface of the thylakoid membrane (i.e. at the O_2 evolving locus), as an incubation is necessary to allow the internal pH to equilibrate after a pH jump. However, the rate of ^{14}C labeling under these experimental conditions is greater at pH 6.0 than it is at pH 6.8, whereas the equilibrium amount of ^{14}C bound (Stemler 1980), as well as the Hill activity (Khanna et al. 1977, Vermaas and van Rensen 1981) is greater at the higher pH. Another explanation for the higher rate of binding at low pH is offered in the model).

That HCO_3^- is indeed the active species was demonstrated conclusively by taking advantage of the pH dependence of the equilibrium ratio of $[CO_2]$ to $[HCO_3^-]$ to effectively hold one concentration constant while varying the other (Blubaugh and Govindjee 1986). The restoration of the Hill activity to HCO_3^- depleted thylakoids was shown to be dependent only on $[HCO_3^-]$ and was independent of $[CO_2]$, $[H_2CO_3^{2-}]$, or $[CO_3^{2-}]$, over the pH range

studied (6.3–6.9), which spanned both sides of the pH optimum. Although these results indicate that HCO_3^- is the binding species, they leave open as a possible role for CO_2 diffusion to the binding site (Sarojini and Govindjee 1981a, b), since they were performed under equilibrium conditions.

The number of binding sites

Stemler (1977) measured the binding of $H^{14}CO_3^-$ to isolated thylakoids and determined that there were two pools of HCO_3^-: a high affinity pool at a concentration of approximately one HCO_3^- per 300–400 chlorophyll molecules and a low affinity pool at a concentration as large or larger than that of the bulk chlorophyll. Depletion of the high affinity pool was correlated with the loss of Hill activity, whereas the role of the low affinity pool, presumed to be largely empty under physiological conditions, remained undetermined.

The low affinity pool. Blubaugh and Govindjee (1984) demonstrated an acceleration of the chlorophyll *a* fluorescence rise in the presence of diuron when excess HCO_3^- was added to non-depleted thylakoids, which they attributed to the binding of HCO_3^- or CO_2 to a very low affinity site separate from the site responsible for activation of the Hill activity. In addition to a large difference in affinity, the authors also observed (1) that the low affinity site appeared to bind HCO_3^- or CO_2 preferentially in the light, whereas the high affinity site binds HCO_3^- preferentially in the dark (see also Stemler and Govindjee 1973, Vermaas and van Rensen 1981), and (2) the high affinity site appeared to be overlaid by diuron (see also Stemler 1977, Vermaas and van renson 1981), which was not the case for the low affinity site.

Other effects of excess HCO_3^- added to non-depleted thylakoids have been reported: (1) it inhibits the photosystem II reduction of silicomolybdate (Barr and Crane 1976); even though (2) it accelerates whole-chain electron transport (Punnet and Iyer 1964, Batra and Jagendorf 1965, Barr and Crane 1976); and (3) it stimulates photophosphorylation (Punnet and Iyer 1964, Batra and Jagendorf 1965). Several of these effects require a significantly larger concentration of HCO_3^- than is required to stimulate the Hill reaction in HCO_3^- depleted thylakoids, suggesting involvement of the low affinity site.

Excess HCO_3^- (or CO_2) has been proposed to effect a conformational change in the coupling factor CF_1 (Cohen and MacPeek 1980). Little is known about this site, and there is the possibility that this effect is non-specific, in that carboxylic acids in general appear to stimulate phosphorylation (Nelson et al. 1972). However, at least one effect of HCO_3^- on CF_1, the increased inhibition of phosphorylation by N-ethylmaleimide in the

presence of HCO_3^- and the decreased ability of adenylates to protect against this inhibition, is specific for HCO_3^- or CO_2 (Cohen and MacPeek 1980).

This effect on CF_1, however, cannot fully explain the stimulation of electron transport by excess HCO_3^-, since this stimulation occurs whether or not ADP and P_i are present (Punnett and Iyer 1964). It is also difficult to rationalize how a conformational change of CF_1 would accelerate the chlorophyll *a* fluorescence rise in the presence of diuron (Blubaugh and Govindjee 1984). Therefore, another low affinity site probably exists. Although HCO_3^-, not CO_2, is the species that restores electron transport to HCO_3^- depleted thylakoids (Blubaugh and Govindjee 1986), the species responsible for these other effects is still an open question.

The high affinity pool. Eaton-Rye (1987) has proposed two HCO_3^- sites in order to explain apparent effects on both PQ binding and protonation of Q_B^-. One site is suggested to be on Fe^{2+} in the Q_A-Fe-Q_B complex of PS II, as proposed by Michel and Deisenhofer (1988), and is responsible for maintaining the proper conformation for efficient PQ binding. The second site is suggested to be more difficult to deplete of HCO_3^- and is responsible for delivering a H^+ to a protein group near Q_B^-.

We have obtained evidence for two HCO_3^- sites, both necessary for Hill activity, from a kinetic study of the dependence on HCO_3^- (Blubaugh 1987): The amount of Hill activity restored to HCO_3^- depleted thylakoids by a half-saturating [HCO_3^-] was non-linear with respect to [Chl], an indication that some endogenous HCO_3^- was present in the depleted samples. Since the basal activity in the absence of any added HCO_3^- was less than 7% of the fully restored rate, it was suggested that the small residual activity commonly observed after HCO_3^- depletion is due to endogenous HCO_3^- not removed during the depletion procedure, and that HCO_3^- is an essential requirement for the Hill activity. When corrected for the endogenous [HCO_3^-], the activity vs. [HCO_3^-] data for these same membranes no longer suggested Michaelis-Menten kinetics, but instead suggested two HCO_3^- sites with cooperative binding ($n > 1.4$).

The large low affinity pool, discussed earlier, does not appear to be correlated with restoration of the Hill activity, as the activity is largely restored even when the low activity pool is mainly empty (Stemler 1977). Therefore, the two cooperative sites suggested to control the Hill activity must be separate from this low affinity pool.

The role of HCO_3^-

Q_B is known to be a transiently bound PQ molecule which, after becoming

doubly reduced to plastoquinol (PQH_2), exchanges with the PQ pool, as was first proposed independently by Velthuys (1981) for green plants and by Wraight (1981) for purple bacteria (ubiquinone replaces PQ in the bacteria). The steps at which protonation of Q_B occurs, however, is not fully elucidated. Diner (1977) proposed, based on thermodynamic considerations of the equilibrium constant for dismutation of duroquinol, compared to the equilibrium constants for electron transfer through the quinones of PS II, that the protonation of Q_B should occur at the level of Q_B^{2-}. Similarly, Pulles et al. (1976) found from the difference absorption spectrum that Q_B^- is unprotonated, and Fowler (1977) observed a binary oscillation in H^+ uptake corresponding to the production of Q_B^{2-}. However, the oscillations observed by Fowler were very small, and were not seen at all by others (Hope and Moreland 1979, Förster et al. 1981, see, however, van Rensen 1988). To account for an unprotonated Q_B^- and a lack of binary oscillation in H^+ uptake, Förster et al. (1981), proposed the protonation of a protein group to stabilize Q_B^-, as was proposed earlier for photosynthetic bacteria (Wraight 1979). Crofts et al. (1984) similarly proposed a scheme in which a protein group near Q_A and Q_B needs to be protonated before Q_B^- can accept a second electron from Q_A^-. They showed that the pKa for this group appears to shift from about 6.4 to approximately 7.9 when Q_B is reduced to Q_B^-, and the oxidation of Q_A^- by Q_B^- is slowed down when this group is unprotonated. This is analogous to what occurs in photosynthetic bacteria, although the pKa's are different (Wraight 1979).

It is tempting to assign HCO_3^- a role in this protonation (*e.g.* Stemler 1977), since the absence of HCO_3^- slows down the reduction of Q_B (Jursinic et al. 1976, Siggel et al. 1977), and the pKa of CO_2/HCO_3^- is about 6.4. The pKa of HCO_3^-/CO_3^{2-} is 10.2 in aqueous solution, though a bound HCO_3^-/CO_3^{2-} would be expected to have a lower pKa, due to stabilization of the negative charge upon binding to a positively charged group, and could be as low as 7.9. Such a speculation has been made (Vermaas 1984, van Rensen 1988). However, it is not likely that HCO_3^- is the group that is undergoing the pKa shift observed by Crofts et al. (1984). For one thing, at pH's below 6.4 the putative protein group is already protonated before Q_B^- formation, and Q_A^- to Q_B^- electron ransfer is not impaired (Crofts et al. 1984). If HCO_3^- is protonated it decomposes to form CO_2, which would leave the HCO_3^- site empty, and electron transfer from Q_A^- to Q_B^- would be impaired. It is possible, however, that HCO_3^- is responsible for providing a ready H^+ to this group when its pKa shifts to 7.9.

Eaton-Rye (1987) measured the kinetics of Q_A^- oxidation after one to ten actinic flashes, as a function of pH, in HCO_3^- depleted thylakoids, and

observed that at pH 7.5 two turnovers of the Q_B "two-electron gate" (i.e. four flashes) were necessary before the maximum inhibition of Q_A^- to Q_B electron flow could be seen on subsequent flashes. By contrast, at pH 6.5 the inhibition was maximum after only one turnover of Q_B (i.e. after two flashes). The maximum inhibition at pH 7.5 was also greater than that at pH 6.5, but this difference was diminished as the flash frequency was increased. To explain these observations, it was suggested that the protonation of the putative protein group near Q_B was the rate-limiting step at alkaline pH when HCO_3^- was not present. This explains the greater inhibition at pH 7.5: at pH 6.5, sufficient H^+s were supposedly available from sources other than HCO_3^- to ameliorate the inhibition, but with increasing flash frequency the time for such a H^+ to arrive became limiting. The greater inhibition at pH 7.5 would also prevent the advancement of the two electron gate during a flash in some centers, to account for the observation that two turnovers of Q_B are necessary to see the full inhibition. (Although HCO_3^- is presumed to supply a H^+ to this protein group, removal of HCO_3^- would not be expected to deprotonate the group.) These centers, then would not be inhibited until the second turnover.

An analysis of the fast phase of Q_A^- oxidation after the first two actinic flashes revealed another pH-dependent impairment, in which Q_A^- to Q_B^- electron flow was slower, not at higher but at lower pH (Eaton-Rye 1987). As the fast phase is presumably due to centers with Q_B already bound prior to the flash (Crofts et al. 1984), this observation was taken to indicate either an impairment in the binding of PQ to the Q_B site (Govindjee and Eaton-Rye 1986, Eaton Rye 1987) or some other unspecified conformational change that is more pronounced at lower pH. Thus in addition to its involvement in the protonation reactions, a second, possibly structural, role for HCO_3^- is implicated (see also Vermaas and Rutherford 1984).

That HCO_3^- depletion may impair PQ binding at the Q_B site was also suggested by the effect of HCO_2^- incubation on the binding of a photoreactive PQ analog, 6-azido-5-decyl-2,3-dimethoxy-p-benzoquinone (6-azido-Q_0C_{10}; Blubaugh 1987). This analog can functionally replace PQ, but becomes covalently attached to the Q_B site during ultraviolet (UV) irradiation (Vermaas et al. 1983). After this covalent attachment, the rise of the Chl *a* fluorescence transient is accelerated (Vermaas et al. 1983, Blubaugh 1987). However, when HCO_2^- was present during the 6-azido-Q_0C_{10}/UV treatment, followed by HCO_3^- addition, the Chl *a* fluorescence rise was not accelerated (Blubaugh 1987). This suggests that HCO_2^- incubation, which is known to remove HCO_3^- from its binding sites, may impair the binding of 6-azido-Q_0C_{10} at the Q_B site.

Physiological significance

Reproducibly large effects of HCO_3^- depletion were not observed in thylakoids until low pH and high salt concentrations were used during the depletion procedure (Stemler and Govindjee 1973). Since 1973, high concentrations of formate, (HCO_2^-) have been routinely used to aid in depleting samples of HCO_3^-. With the measurement by several laboratories of a dissociation constant for the HCO_3^- * PS II complex of 80 μM (Stemler and Murphy 1983, Snel and van Rensen 1984, Jursinic and Stemler 1986), it has been suggested that the stimulatory effect of HCO_3^- is no more than a simple reversal of an inhibitory effect of HCO_2^- (Stemler and Murphy 1983). However, the argument was based on the observation that the intracellular [CO_2] is around 5 μM in photosynthesizing chloroplasts (Hesketh et al. 1982). It is now established that HCO_3^-, not CO_2, is the activating species (Blubaugh and Govindjee 1986). At a pH of 8 (the approximate stromal pH during photosynthesis), the [HCO_3^-] in equilibrium with 5 μM CO_2 would be 220 μM. Also, the Kd value of 80 μM is overestimated by at least a factor of two, since its determination did not take into account the fact that nearly half of the added HCO_3^- was converted into CO_2. Thus, the calculated Kd should be around 40 μM. Therefore, there is no reason to presume that the activating sites are empty under physiological conditions. On the other hand, while a HCO_3^- dependence in the absence of HCO_2^- has been observed (Good 1963, Robinson et al. 1984, Eaton-Rye et al. 1986, see also chapter 2 in Blubaugh 1987), the effect is never as dramatic as when inhibitory anions are present. This does not, however, necessarily imply that HCO_3^- is not required – it could simply reflect the difficulty of removing HCO_3^- without the assistance of a competitor. It has recently been suggested, from a kinetic study of the HCO_3^- effect, that HCO_3^- is an essential activator and that the small amount of activity remaining after HCO_3^- depletion is due to endogenous HCO_3^- that was not removed (Blubaugh 1987).

The observation of a HCO_3^- effect on PS II *in vivo* is difficult to distinguish, due to the obvious requirement for CO_2 in the Calvin cycle. Nevertheless, Ireland et al. (1987) have attempted to do this in leaves by examining the effect on chlorophyll *a* fluorescence of a small decrease in the intracellular CO_2 concentration from an already low level. Although CO_2 fixation decreased slightly from an already low level, they observed a much larger increase in [Q_A^-], and concluded that HCO_3^- is involved in the quinone reactions *in vivo*. (Also see Garab et al. 1983.)

In the model which follows, HCO_3^- is assumed to be a physiological requirement; CO_2 is a diffusing species.

The model

In this working model (see Fig. 2), one HCO_3^- (Site A, see Table 1) is a bidentate ligand to the Fe^{2+}, as proposed by Michel and Deisenhofer (1988), and forms a salt bridge necessary for the functional configuration of the reaction center. Perhaps, it is H-bonded to a residue on the D_2 protein, as a replacement for glutamate-232 of the M subunit of the bacterial reaction center (Michel and Deisenhofer 1988). Disruption of this salt bridge is suggested to alter the distance between Q_A and Q_B, resulting in a slower electron transfer, to make Q_A^- more accessible to direct oxidation, and to alter the binding affinity of plastoquinone to the Q_B site.

We propose that a second high-affinity HCO_3^- (Site B, see Table 1) is bound to an arginine in the D_1 protein, as suggested by Shipman (1981), and is involved in protonating a histidine at the Q_B site. The histidine would be the group whose pKa has been observed to shift from 6.4 to 7.9 upon

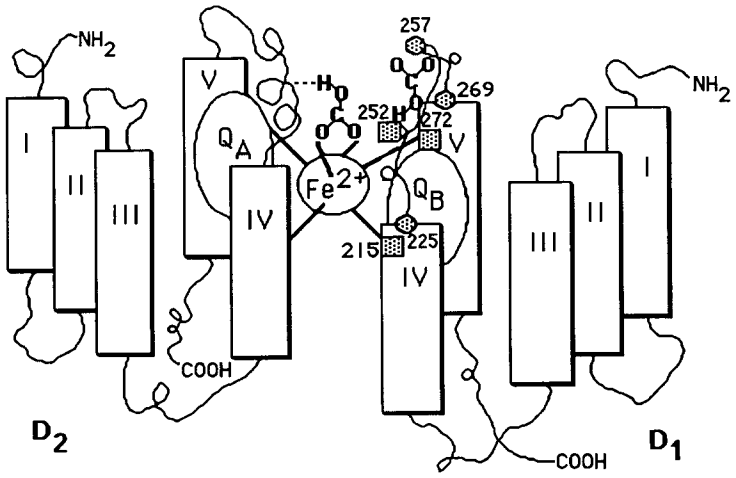

Fig. 2. A model of the Q_A-Fe-Q_B complex of the D_1-D_2 reaction center proteins, showing the HCO_3^- ligand to Fe^{2+} (site A, see text) and the possible arginine/histidine pairs on D_1 that comprise the second high-affinity HCO_3^- site (site B, see text). At site A, the HCO_3^- is shown H-bonded to a residue on the D_2 protein, as a structural requirement for the native configuration of the reaction center. In photosynthetic bacterial reaction centers, which do not appear to exhibit a HCO_3^- dependence (RJ Shopes, D Blubaugh, CA Wraight and Govindjee, unpublished), such a bridge is supplied by glutamate-232 of the M subunit (Michel and Deisenhofer 1986). At site B, the HCO_3^- is bound to an arginine and undergoes acid/base chemistry with a nearby histidine. The possible arginines are indicated by the stippled circles (arginines 225, 257, 269), the possible histidines by stippled squares (histidines 215, 252, 272); arginine 257/hisitidine 252 are the most likely pair (see text). The locations of these residues are patterned after the folding sequence of Trebst (1987). The rectangles represent the membrane spanning regions of the proteins.

formation of Q_B^- (Crofts et al. 1984). This pKa shift, induced by the negative charge on Q_B^-, encourages protonation of the histidine by the HCO_3^-, whose own pKa is lowered by the electron withdrawing effects of the arginine (Fig. 3). The possible arginines are indicated by the stippled circles in Fig. 2 (Arg 225, 257, 269); the possible histidines are indicated by the stippled squares (His 215, 252, 272). The locations of these residues are patterned after the folding sequence suggested by Trebst (1987). There are thus three arginine/histidine pairs that could be involved. In two of these (Arg 257/His 252 and Arg 269/His 272), the arginine and histidine would be separated by a single helical turn, while in the third (Arg 225/His 215) they would be separated by two helical turns. Since histidines 215 and 272 are already liganded to Fe^{2+}, we consider that histidine-252 is most likely to undergo the acid/base chemistry proposed here. Therefore, we favor **arginine-257** and **histidine-252** as the catalytic site (Site B) for this second HCO_3^-. We further suggest that the HCO_3^- ions at Site A and Site B bind cooperatively.

The CO_3^{2-} resulting after deprotonation of the HCO_3^- at Site B is suggested to bind less tightly because of the greater delocalization of the charge over the molecule (Fig. 3, see also Blubaugh and Govindjee 1986). Another HCO_3^- from an intramembrane pool displaces CO_3^{2-}, ensuring irreversibility of the protonation reaction. Interaction of the new HCO_3^- with the N of histidine may favor H^+ transfer from histidine to Q_B^-. CO_3^{2-}, with a pKa of 10.2, would readily pick up a H^+.

A large number of low-affinity sites (Site C, see Table 1; see also Stemler 1977, Blubaugh and Govindjee 1984) may act as a buffer of the intrathylakoid [HCO_3^-], to keep the arginine loaded during rapid turnover of the reaction center. Consumption of H^+ during illumination would drive the equilibrium toward HCO_3^-, leading to a net influx of CO_2 (and an effective influx of H^+) and loading of the low-affinity sites (Fig. 4). Efflux of HCO_3^- (as CO_2) in the dark would be limited by the availability of H^+. Low pH and high ionic strength, both necessary for effective HCO_3^- depletion, are suggested to disrupt the $Fe^{2+}.D_2$ salt bridge (Site A) and to expose the low affinity sites to the bulk phase (Fig. 5).

Basic assumptions of the model and their justifiction

Assumption 1: HCO_3^- is the active species. It was demonstrated previously (Blubaugh and Govindjee 1986) that restoration of the Hill activity to HCO_3^- depleted thylakoids depends only on the equilibrium [HCO_3^-]; it is independent of the equilibrium [CO_2], [H_2CO_3] or [CO_3^{2-}].

Assumption 2: There are two cooperative sites of HCO_3^- binding. Although it has been previously reported that the restoration of the Hill activity to

Table 1. Proposed sites of HCO_3^--binding in photosystem II, their functions and supporting evidence. For details, see text.

Site	Location	Function	Supportive arguments
A	Ligand to Fe^{2+}, H-bonded to residue on D_2 protein	● Structural; salt bridge holds reaction center in proper conformation ● Disruption of salt bridge slows electron transfer from Q_A^- to Q_B, impairs PQ binding, permits direct oxidation of Q_A^-, allows exchange of HCO_3^- pool (sites C) with bulk phase ●Cooperative binding with site B	● EPR signal at g = 1.82 increases dramatically upon HCO_3^- depletion (Vermaas & Rutherford, 1984) ● Formate prevents oxidation of Fe^{2+} by exogenous quinones (Zimmermann & Rutherford, 1986) ● In bacterial reaction centers, glutamate-232 of M subunit is bidentate ligand to Fe^{2+}; the region containing this residue is deleted in D_2 protein of plants (Michel & Deisenhofer, 1988) ●Two possible sites of cooperative binding; endogenous HCO_3^- possibly exchanges with bulk phase at low pH, high ionic strength (Blubaugh 1987) ●In presence of formate, azido analog of PQ becomes less attached to Q_B site; may also oxidize Q_A^- directly (Blubaugh 1987)
B	D_1 protein, at one of following arginine/histidine pairs: Arg-225/His-215 Arg-257/His 252* Arg-269/His-272 *The pair favored in this model	● Protonation of Q_B^- ●HCO_3^- provides rapid source of H^+ to histidine to stabilize Q_B^- ●Released CO_3^{2-} is recipient of H^+ ●Cooperative binding with site A	●Arginine-HCO_3^- complex would have proper dipole moment for herbicide binding (Shipman, 1981) ●The herbicide diuron overlays HCO_3^- (Stemler, 1977, Blubaugh and Govindjee, 1984) ●Bromoacetate modifies a residue (histidine, cysteine or tyrosine) near HCO_3^- binding site (Stemler, 1985)

C	Large number of low affinity sites in thylakoid membrane, in equilibrium with site B	• Not saturated, ordinarily • Provides pool of HCO_3^- for rapid replacement of CO_3^{2-} at site B, after protonation of histidine • Buffers intrathylakoid [HCO_3^-]	• Large pool of low affinity $H^{14}CO_3^-$ binding sites (Stemler, 1977) • High concentrations of HCO_3^- stimulate electron transport (Punnett & Iyer, 1964; Barr & Crane, 1976) and accelerate Chl *a* fluorescence rise in presence of diuron (Blubaugh & Govindjee, 1984); these effects are due to a separate site of HCO_3^- action (Batra & Jagendorf, 1965; Blubaugh & Govindjee, 1984)

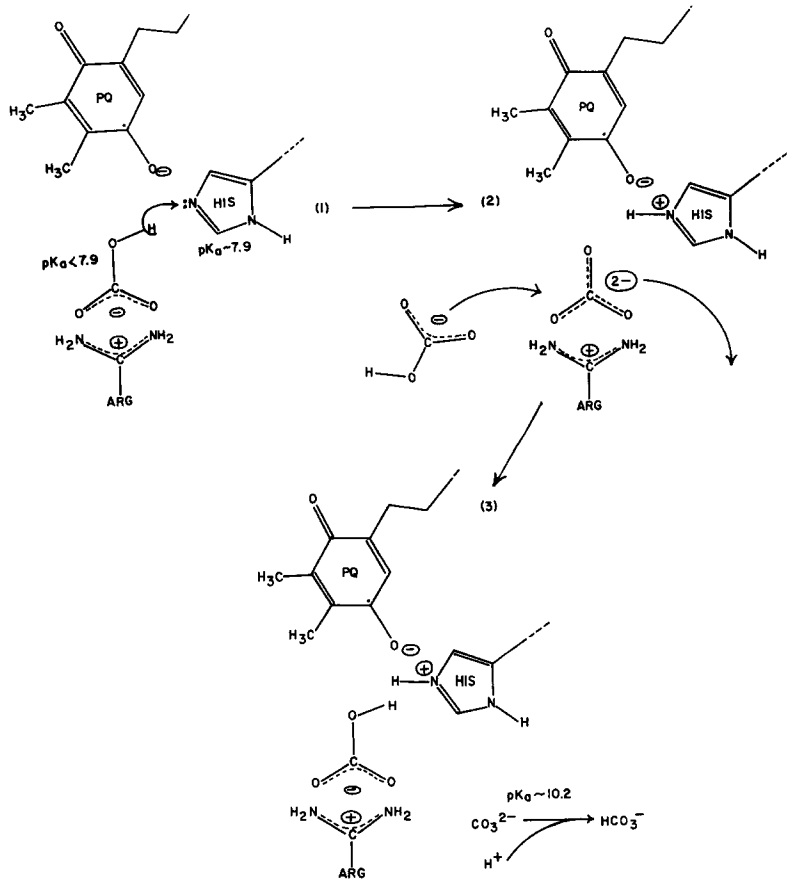

Fig. 3. A working model for the involvement of HCO_3^- in the protonation of Q_B^-. Step 1: (start at top left) When Q_B (PQ) is reduced to Q_B^-, the pKa of the histidine (HIS) shifts from 6.4 to 7.9 (Crofts et al. 1984), in response to the repulsive interaction between the negative charge on Q_B^- and the lone pair of electrons on histidine. The pKa of the bound HCO_3^- is presumed to be lowered from 10.2 to about 7.9, due to the electron withdrawing effect of the arginine (ARG). Therefore, there would be an equilibrium sharing of a H^+ between CO_3^{2-} and histidine. Step 2: (See top right) Replacement of CO_3^{2-} by another HCO_3^-, would drive the equilibrium toward histidine and ensure irreversibility of the protonation reaction. Step 3: (see middle and bottom) The pKa of the free CO_3^{2-} is 10.2, so reprotonation would be very rapid. Steric effects between the OH of HCO_3^- and the H on histidine would favor the transfer of H^+ from histidine to Q_B^- or Q_B^{2-},

HCO_3^- depleted thylakoids exhibits Michaelis-Menten kinetics (e.g. Snel and van Rensen 1983), we have recently observed that even in well-depleted thylakoids exhibiting a large HCO_3^- dependence (basal activity < 7% of the fully restored rate), a significant amount of endogenous HCO_3^- remains (Blubaugh 1987). When the kinetic data was corrected for the estimated

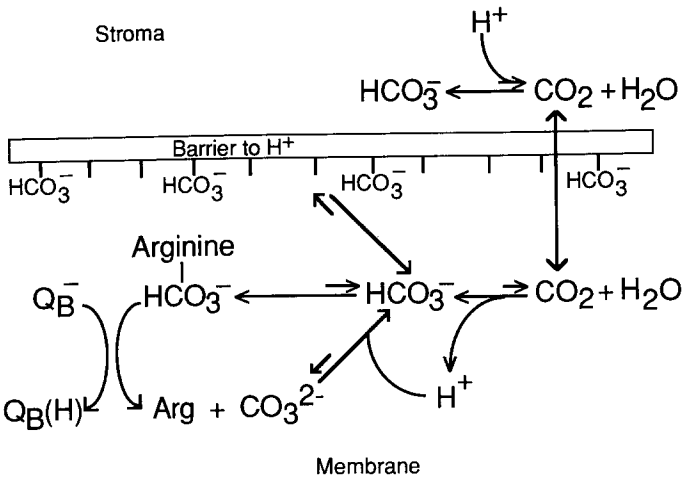

Fig. 4. A working model for the involvement of a sequestered intrathylakoid pool of HCO_3^- in the protonation of Q_B^-. The membrane provides an effective barrier to H^+ and HCO_3^- diffusion. CO_2 diffuses (see Sarojini and Govindjee, 1981a,b) into the sequestered space, driven by the consumption of H^+ as Q_B is turned over. Free HCO_3^- is in equilibrium with the high-affinity binding site on the arginine near Q_B (site B, see text), with the intracellular [CO_2], with free CO_3^{2-}, and with a large number of postulated low affinity sites (sites C, see text), represented here as small tic marks. The longer arrows indicate the direction in which equilibrium would lie at a pH of 8. In the stroma, H^+ is taken up in the conversion of HCO_3^- to CO_2, and is effectively transported by the diffusion of CO_2. The low affinity sites buffer the intrathylakoid [HCO_3^-]. Although their affinity is low, there is a large number of them, so many HCO_3^- molecules are bound. In the dark, efflux of the HCO_3^- pool would be limited by the availability of H^+ for conversion of HCO_3^- to CO_2.

endogenous [HCO_3^-], a Hill coefficient of $N_{app} > 1.4$ was obtained, indicating at least two sites with cooperative binding.

Assumption 3: A bidentate ligand to Fe^{2+} exists (Site A). HCO_3^- depletion has been observed to induce a large increase in the EPR signal at g = 1.82 (Vermaas and Rutherford 1984), attributed to the Q_A^-* Fe^{2+} complex (Rutherford and Zimmermann 1984). In bacterial reaction centers, the Fe^{2+} forms two ligands to the carboxyl group of a glutamate residue on the M subunit and one ligand to each of four histidines, two of which are in the L subunit, and two of which are in the M subunit (Michel et al. 1986). In PS II reaction centers, the Q_B protein, D_1, has a high degree of similarity with the L subunit, while the D_2 protein (to which D and Q_A are believed to bind) is highly similar to the M subunit (e.g. Trebst and Draber 1986, Trebst 1987). However, one major difference is that D_2 is lacking an extra loop which, in the M subunit, carries the glutamate ligand to the Fe^{2+}. Michel and Deisenhofer (1988) have suggested that in PS II, HCO_3^- takes the place of the

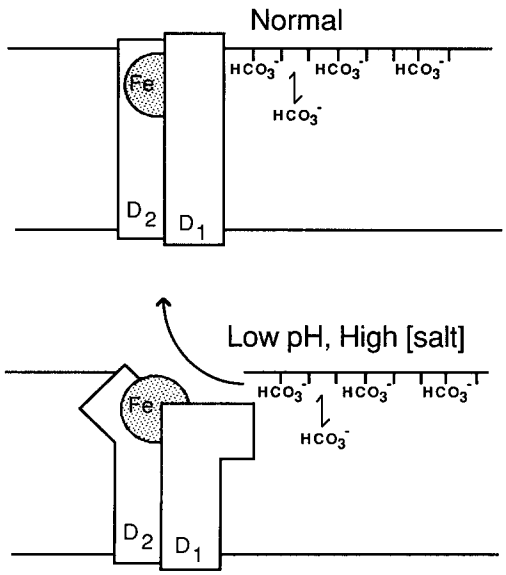

Fig. 5. A model of the D_1-D_2 reaction center proteins and intrathylakoid HCO_3^- pool. At low pH and high ionic strength, a conformational change exposes the low affinity HCO_3^- sites (sites C, see text) to the bulk phase. The conformational change may be due to disruption of a Fe^{2+}-HCO_3^--D_2 salt bridge (Fig. 1).

glutamate ligand. This would explain why a HCO_3^- dependence has never been observed in the photosynthetic bacteria (R.J. Shopes, D. Blubaugh, C.A.Wraight and Govindjee, unpublished). To complete the analogy, the HCO_3^- in PS II would presumably form a salt bridge between the Fe^{2+} and some residue in the D_2 protein.

Assumption 4: A binding site at an arginine near Q_B exists (Site B). One HCO_3^- site appears to be overlaid by the herbicide diuron. Diuron prevents the effect of HCO_3^- on the variable Chl *a* fluorescence in HCO_3^- depleted thylakoids if it is added first, but it does not reverse the effect of HCO_3^- if it is added second (Blubaugh and Govindjee 1984). Diuron in believed to replace PQ from the Q_B binding site (e.g. Oettmeier and Soll 1983). One thing that PS II herbicides appear to have in common is a flat polar component with a dipole moment of 3–5 Debyes (Shipman 1981). Thus, Shipman (1981) proposed that the herbicides bind electrostatically to a strongly polar binding site within a hydrophobic surface on the Q_B protein. HCO_3^-, bound to an arginine, could provide an appropriate electric field for such an interaction, and was considered by Shipman to be a likely part of the herbicide binding environment. The observation that diuron appears to

overlay the HCO_3^- in PS II (Stemler 1977, Blubaugh and Govindjee 1984, Eaton-Rye and Govindjee 1988b), supports this notion.

Assumption 5: Acid/base chemistry occurs with a histidine near Site B . Because of the very close proximity of this HCO_3^- to Q_B, and because a protein group near Q_B is protonated upon reduction of Q_B to Q_B^- (Crofts et al. 1984), it is likely that the hydroxyl group of this HCO_3^- is involved in an acid/base reaction with the protein group. Eaton-Rye (1987) has also suggested that HCO_3^- is required at this site for the protonation of a site to stabilize Q_B^-. In our model, a histidine is shown as the protein group to be protonated for three reasons (1) the observed pKa is 6.4 (Crofts et al. 1984); (2) there is a histidine cluster around the Q_B site (Michel et al. 1986, Trebst and Draber 1986); and (3) bromoacetate, a protein modifier (histidine, lysine, tyrosine, and cysteine residues), which competes with HCO_3^- for its binding site, becomes covalently attached to a residue near the site (Stemler 1985). The pKa of this group shifts to about 7.9 upon formation of Q_B^- (Crofts et al. 1984). The negative charge of Q_B^- in close proximity to the histidine would cause such a shift in the pKa by stabilizing the positive charge of the protonated histidine. The pKa of the hydroxyl group of HCO_3^- is 10.2 in aqueous solution, but because of the electron withdrawing effect of the arginine, its pKa would be shifted lower. Thus, upon reduction of Q_B to Q_B^-, a H^+ transfer could occur from HCO_3^- to the histidine.

Assumption 6: CO_3^{2-} dissociates from Site B to be replaced by a HCO_3^- from an intrathylakoid pool. Blubaugh and Govindjee (1986) suggested that CO_3^{2-} binds less tightly than HCO_3^-. This was inferred from the difference in the extent of inhibition by nitrite (NO_2^-) versus nitrate (NO_3^-) (e.g. Stemler and Murphy 1985), which closely resemble HCO_3^- and CO_3^{2-}, respectively, in their electronic structures. Thus, it is presumed that the CO_3^{2-} resulting after H^+ transfer is displaced by another HCO_3^-. Although CO_3^{2-} has a greater negative charge for interaction with the arginine at Site B, a significant portion of the charge is distributed away from the arginine, toward, in our model, the negative charge on Q_B^-, which we would expect to be destabilizing. In the case of HCO_3^-, however, all of the charge would be directed toward the arginine. Although it is possible that the CO_3^{2-} could remain in place and simply pick up a H^+ from another source, there would be a distinct advantage to the system if CO_3^{2-} were to be replaced as proposed. Once released from its site, the pKa of CO_3^{2-} would return to 10.2, and reprotonation would be very rapid. Furthermore, the rapid replacement of CO_3^{2-} with HCO_3^- would ensure that the H^+ remains on the histidine; the equilibrium sharing of a H^+ between HCO_3^- and histidine would be shifted

far in favor of the histidine. Such an exchange will occur most readily if a pool of HCO_3^- is available. The uptake of a H^+ from the bulk phase could, of course, occur with any of the CO_3^{2-} molecules in the pool and would be faster than if the CO_3^{2-} had to diffuse to the outer surface.

Assumption 7: A large number of low affinity sites exist (Sites C). Stemler (1977) demonstrated the existence of a large low affinity pool of HCO_3^-. This HCO_3^- could be the intrathylakoid pool proposed here. However, the number of HCO_3^- "sites" was found to at least approach, and perhaps exceed, the number of chlorophyll molecules (Stemler 1977). Similarly, Blubaugh (1987), from kinetic evidence for endogenous HCO_3^- remaining in HCO_3^- depleted thylakoids, estimated that perhaps as many as several thousand HCO_3^- molecules per PS II remained non-exhangeable with the bulk phase at moderate pH. From consideration of the possible size of the intrathylakoid $[HCO_3^-]$, which must remain in equilibrium with the intracellular $[CO_2]$, a large number of low affinity binding sites is proposed. By this means, a large number of intrathylakoid HCO_3^- molecules could exist at a moderate free $[HCO_3^-]$. The advantage to the system would be the buffering of the free $[HCO_3^-]$ against a drop in the intracellular $[CO_2]$ during photosynthesis. Although we show the low affinity sites binding HCO_3^-, CO_2 could also be bound as carbamate complexes with protein amino groups.

Assumption 8: A barrier to H^+ and HCO_3^- diffusion exists. The proteinaceous environment around Q_B has long been known to be a barrier to H^+ diffusion (e.g. Renger 1976). CO_2, rather than HCO_3^- appears to be the species which diffuses to the active sites (Sarojini and Govindjee 1981a, 1981b).

Discussion of the model

This model proposes two high affinity sites of HCO_3^- binding (Sites A and B) and numerous low affinity sites (Sites C). The HCO_3^- at Site A forms a salt bridge between the Fe^{2+} and a residue on the D_2 reaction center protein (Fig. 2), and is probably an essential requirement. The HCO_3^- at Site B is bound to an arginine (most likely arginine-257) of the D_1 reaction center protein and protonates a histidine (most likely histidine-252) in D_1, as part of a mechanism for protonation of Q_B^- (Fig. 3).

Such a specific proposal allows the application of molecular genetics tools (such as site-directed mutagenesis) to be used to test our model. After losing a H^+ to the histidine, the CO_3^{2-} at Site B exchanges with a HCO_3^- from an intrathylakoid pool. The low affinity Sites C buffer the intrathylakoid $[HCO_3^-]$ to ensure a rapid exchange with CO_3^{2-} at Site B, and perhaps to protect against fluctuations in the intracellular $[CO_2]$ (Fig. 4).

Neither CO_2, H_2CO_3 nor CO_3^{2-} have any apparent effect on the restoration of the Hill activity by HCO_3^- (Blubaugh and Govindjee 1986). Thus, the active species is presumed to be HCO_3^- at both sites A and B. Since the inhibitory formate (HCO_2^-) is identical to HCO_3^- in all respects except for a missing hydroxyl group, it can be presumed that the hydroxyl group is important for functioning at both sites. At Site A the hydroxyl group is suggested to be involved in an H-bond with a residue on the D_2 protein, to provide the proper conformation to the PS II reaction center. This salt bridge would be adversely affected by protonation during a low pH treatment, or by shielding of its charge by high salt concentrations. Blubaugh (1987) suggested that some ionizable group, when protonated or charge shielded, causes the release of otherwise-held HCO_3^-. It is possible that low pH or high ionic strength, both of which facilitate HCO_3^- depletion, disrupts the Fe^{2+}-HCO_3^--D_2 salt bridge, inducing a conformational change which permits the exchange of the intramembrane pool of HCO_3^- with the bulk phase (Fig. 5). Thus, low pH and high ionic strength alone are likely to induce a partial HCO_3^- depletion by permitting the HCO_3^- in the vicinity of Q_B to exchange with a large volume.

Although HCO_3^- was recently shown to be the binding species (Blubaugh and Govindjee 1986), Stemler (1980), earlier, concluded that CO_2 is the active species and that it binds on the lumen side of the membrane, because the rate of $H^{14}CO_3^-$ binding was pH independent for the first few minutes after a pH jump, but then showed a marked pH dependence after a 5 min incubation at the new pH, with the binding being greater at lower pH values. All that is necessary to reconcile this experiment with the model presented here is to postulate that the salt bridge is disrupted by low pH on a time scale of minutes or that the diffusion of HCO_3^- out of the sequestered space is slow. Immediately after a jump to low pH, the HCO_3^- binding sites would still be sequestered. After an incubation at low pH, however, the sites would become exchangeable with the bulk phase, and the rate of $H^{14}CO_3^-$ binding would be accelerated.

The pH optimum of the HCO_3^- effect is about 6.5 (Khanna et al. 1977, Vermaas and van Rensen 1981). The ascending arm is probably due to the fact that HCO_3^- is the active species (Blubaugh and Govindjee 1986), and the pKa of CO_2/HCO_3^- is about 6.35. The descending arm may reflect the pKa of the group to which HCO_3^- is H-bonded in the salt bridge. This pKa would be about 6.8, suggesting a histidine residue. At pH values significantly below this, both the removal and the binding of HCO_3^- would be facilitated (see Stemler 1977). When the conformational change occurs, the affinity for HCO_3^- at Site B would also be lowered because of cooperativity between Sites A and B (Blubaugh 1987).

At Site B, the hydroxyl group on HCO_3^- is suggested to undergo acid/base chemistry with a histidine. Stemler (1985) showed that bromoacetate, a protein modifier, as well as a structural analog of HCO_3^-, binds covalently in PS II, and that prior to covalent attachment it inhibits $H^{14}CO_3^-$ binding competitively, whereas after covalent attachment $H^{14}CO_3^-$ binding is inhibited noncompetitively. The model presented here is consistent with this observation: Bromoacetate undergoes a nucleophile substitution reaction with histidine, lysine, tyrosine and cysteine residues (Korman and Clarke 1955). Ordinarily, the reaction is very inefficient, but it can be accelerated considerably if the amino acid is "activated"; for instance, the half-time of the reaction is about 12 h with free histidine (Korman and Clarke 1955), as opposed to less than 1 h with a histidine in ribonuclease (Barnard and Stein 1959). The reaction proceeds significantly within several hours in PS II, and is faster in the light (Stemler 1985). Structurally, the C-Br bond of bromoacetate corresponds to the O-H bond of HCO_3^-. The orientation of this bond towards a histidine, as well as the pKa shift of the histidine upon Q_B^- formation, would, by increasing the nucleophilicity of the histidine, increase the reaction rate. After covalent attachment (Br is replaced by the histidyl residue), the carboxyl end would be pulled away from the arginine, but would remain close enough to continue to noncompetitively inhibit HCO_3^- binding at Site B.

Site B is very close to the site of Q_B binding, such that diuron binding to the Q_B site can also overlay the HCO_3^- site (Stemler 1977, Blubaugh and Govindjee 1984). Stemler and Murphy (1983) reported that diuron binding eliminates half of the HCO_3^- binding sites, consistent with this model (Site B eliminated, but not Site A). It has been similarly shown previously that HCO_3^- depletion lowers the binding activity of diuron-type herbicides (Khanna et al. 1981) and *vice versa* (van Rensen and Vermaas 1981, Vermaas et al. 1982, Snel and van Rensen 1983). This interaction is only partially competitive, and was explained by Vermaas and van Rensen (1981) as due to an overlapping of sites.

Since the two high affinity HCO_3^- sites (Sites A and B) are probably cooperative, anything that affects the binding at one site would be expected to affect the binding at the other through allosteric interactions. Thus, diuron would be expected to alter the binding affinities of HCO_3^- at both sites. Stemler and Murphy (1984) reported that the binding of atrazine to a high affinity site (i.e. the Q_B site) seems to remove some HCO_3^- "neither competitively nor noncompetitively but somehow directly". Similarly, the binding of HCO_3^- at site A would be expected to affect diuron binding, even though the sites are spatially separated. The apparent ability of silicomolybdate (SiMo) to reduce the binding affinity of diuron (Böger 1982, Graan

1986) may be due to SiMo removing HCO_3^- from the Fe^{2+} (Site A), rather than due to competition between SiMo and diuron for the same site, as was suggested by Böger (1982). That SiMo does remove bound HCO_3^- was shown previously by Stemler (1977), who observed that when the diuron was added before the SiMo, about half as much HCO_3^- was removed. This latter observation is predicted by this model; by overlaying site B, diuron would prevent the dissociation of half of the high-affinity HCO_3^-.

HCO_3^- may be required at Site A to help hold the reaction center proteins D_1 and D_2 together, via the Fe^{2+}. Removal of this HCO_3^- would be expected to induce a significant conformational change in PS II, which could disrupt electron flow through the quinones. Allostery is most often associated with polymeric enzymes. The involvement of HCO_3^- in a salt bridge to maintain the active configuration of D_1 and D_2 is, therefore, consistent with the suggested cooperativity (Blubaugh 1987) between the two HCO_3^- sites (Sites A and B). To fully explain the cooperativity, HCO_3^- binding at the arginine near Q_B (Site B) should also induce a conformational change that brings D_1 and D_2 closer to their native structure, thereby favoring the binding of HCO_3^- at Fe^{2+} (Site A).

Recently, it has been shown that some exogenous quinones, when reduced to their semiquinone form, can extract their second needed electron from the Fe^{2+}, and that formate blocks this oxidation of the Fe^{2+} (Zimmermann and Rutherford 1986). It is plausible that by removing the HCO_3^- ligand to the Fe^{2+} (Site A), formate induces a conformational change that increases the distance between the Fe^{2+} and the Q_B site, thus making electron transfer less likely. This same conformational change may then allow the exogenous quinones to accept electrons directly from Q_A^- (see Blubaugh 1987). SiMo, which might act as a metal chelator, may have a similar effect; by removing HCO_3^- from Site A, it would be able to both expose Q_A^- and act as the electron acceptor. The existence of such a conformational change upon addition of SiMo was suggested previously (Zilinskas 1975). Excess bicarbonate inhibits the photosynthetic reduction of SiMo (Barr and Crane 1976), perhaps by simple competition for the liganding site on the Fe^{2+}. Such a conformational change might also slow electron transfer from $Pheo^-$ to Q_A, as was recently observed for disulfiram (tetraethylthiuramdisulfide), which, as a metal chelator, was suspected of binding to the Fe^{2+} (Blubaugh and Govindjee 1988).

Q_{400}, first identified as a high-potential component of PS II by Ikegami and Katoh (1973), and which is now known to be the Fe^{2+} (Petrouleas and Diner 1986), is oxidized when chloroplasts are incubated with ferricyanide (FeCy) in the presence of diuron (Ikegami and Katoh 1973, Wraight 1985). Like the deceleration of the chlorophyll *a* fluorescence rise in the presence

of diuron in HCO_3^- depleted thylakoids (Vermaas and Govindjee 1982b, Blubaugh and Govindjee 1984), the oxidation of Q_{400} by FeCy in the presence of diuron is also dependent on the order of addition (Ikegami and Katoh 1973, Bowes et al. 1979). Although the oxidation of Q_{400} can be partially observed when diuron is added first, under conditions involving the unstacking and then restacking of the grana (Wraight 1985), observation of the full effect requires that FeCy be added before the diuron. This suggests that FeCy, in addition to acting as a PS I electron acceptor, may be binding to the HCO_3^- site near Q_B (Site B). It may oxidize the Fe^{2+} from this position or, alternatively, removal of HCO_3^- from this site may permit Q_B^- to oxidize the Fe^{2+}. The ability of some exogenous quinones to oxidize Fe^{2+} from the Q_B site when they become reduced to the semiquinone (Zimmermann and Rutherford 1986) may likewise be due to an alteration in the binding affinity of HCO_3^- at Site B by these exogenous quinones. Although FeCy may bind to the HCO_3^- site near Q_B (Site B), it could be exerting an effect on the Fe^{2+} allosterically through the HCO_3^- site on the Fe^{2+} (Site A). FeCy is also able to oxidize Q_A^- directly in the presence of diuron at high salt concentrations (Itoh 1978) or at low pH (Itoh and Nishimura 1977), two treatments which, as discussed earlier, are likely to remove the liganding HCO_3^- from the Fe^{2+}. This supports the proposition above that a conformational change induced by the removal of HCO_3^- from one site permits the direct oxidation of Q_A^- by exogenous acceptors at the Q_B site. High salt concentrations actually decrease the oxidation of Q_A^- by FeCy when the pH is already low (Itoh 1978). This is understandable with the above model: when HCO_3^- is already gone from site A, high salt will have no additional effect, other than perhaps a competition with the FeCy for site B.

The affinity of the Q_B site for PQ may be lowered by the removal of HCO_3^- (Blubaugh 1987, see, also, Eaton-Rye 1987). This could be due to effects of HCO_3^- at either site A or B. Eaton-Rye (1987) has speculated that HCO_3^- binding at the Fe^{2+} (site A) affects PQ binding, while another HCO_3^- near Q_B (Site B) is involved in the protonation of Q_B^-. If HCO_3^- binding tightens PQ binding, it follows necessarily that PQ binding also tightens HCO_3^- binding. This may be sufficient to explain the preferential binding of HCO_3^- in the dark as opposed to the light (Stemler and Govindjee 1973, Stemler 1979), particularly when formate is present (Vermaas and van Rensen 1981). In HCO_3^- depleted thylakoids, Q_B will be largely reduced in the light (PQ^{2-}) and slow to exchange with the PQ pool. The absence of oxidized PQ in the Q_B site would make the affinity for HCO_3^- at site A less than it would be in the dark. While formate has a carboxyl group that could be a ligand to Fe^{2+} (Site A), it would not be able to H-bond with D_2, so it would be ineffective at providing the salt bridge that HCO_3^- can provide. Thus, formate may

compete with HCO_3^- for Site A without having the same cooperativity with PQ binding (formate would also not have the same cooperativity with another formate at site B; this might explain why a relatively small [HCO_3^-] (e.g. 10 mM) can outcompete a much larger concentration of formate (e.g. 100 mM). Therefore, in the presence of formate the difference in HCO_3^- binding affinity in the light versus dark would be even more significant, since the HCO_3^- would have the added hindrance of a competitor whose binding is uninfluenced by the redox state of Q_B.

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