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Photosystem II and the unique role of bicarbonate: A historical perspective

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Abstract

In photosynthesis, cyanobacteria, algae and plants fix carbon dioxide (CO_2) into carbohydrate; this is necessary to support life on Earth. Over fifty years ago, Otto Heinrich Warburg discovered a unique stimulatory role of CO_2 in the Hill reaction (i.e., O_2 evolution accompanied by reduction of an artificial electron acceptor), which, obviously, does not include any carbon fixation pathway; Warburg used this discovery to support his idea that O_2 in photosynthesis originates in CO_2. During the 1960s, a large number of researchers attempted to decipher this unique phenomenon, with limited success. In the 1970s, Alan Stemler, in Govindjee's lab, perfected methods to get highly reproducible results, and observed, among other things, that the turnover of Photosystem II (PSII) was stimulated by bicarbonate ions (hydrogen carbonate): the effect would be on the donor or the acceptor, or both sides of PSII. In 1975, Thomas Wydrzynski, also in Govindjee's lab, discovered that there was a definite bicarbonate effect on the electron acceptor (the plastoquinone) side of PSII. The most recent 1.9 Å crystal structure of PSII, unequivocally shows HCO_3^- bound to the non-heme iron that sits in-between the bound primary quinone electron acceptor, Q_A, and the secondary quinone electron acceptor Q_B. In this review, we focus on the historical development of our understanding of this unique bicarbonate effect on the electron acceptor side of PSII, and its mechanism as obtained by biochemical, biophysical and molecular biological approaches in many laboratories around the World. We suggest an atomic level model in which bicarbonate/carbonate plays a key role in the protonation of the reduced Q_B. In addition, we make comments on the role of bicarbonate on the donor side of PSII, as has been extensively studied in the

labs of Alan Stemler (USA) and Vyacheslav Klimov (Russia). We end this review by discussing the uniqueness of bicarbonate's role in oxygenic photosynthesis and its role in the evolutionary development of O_2-evolving PSII.

Keywords

Bicarbonate (hydrogen carbonate) effect, electron transport, protonation reactions, O_2 evolution, Photosystem II

Abbreviations

CA, carbonic anhydrase; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenil)-1,1-dimethylurea (diuron); DPC, 1,5-diphenylcarbazide; HCO_3^-, hydrogen carbonate (bicarbonate) ion; MS, mass spectrometry; NHI, non-heme iron; OEC, oxygen-evolving complex; P680, primary electron donor molecule (Chl) in photosystem II; P700, primary electron donor molecule (Chl) in photosystem I; Pheo, pheophytin; PQ, plastoquinone; PQH_2, plastoquinol; PSI, photosystem I; PSII, photosystem II; Q_A, primary quinone electron acceptor of PSII; Q_B, secondary quinone electron acceptor of PSII; RC, reaction center; S_i, redox state of the OEC, where i is the number of stored oxidizing equivalents; TL, thermoluminescence

1. Introduction

1.1. Role of inorganic carbon and its interconversion in living organisms

Carbon dioxide (CO_2) is not only a greenhouse gas in the Earth's atmosphere, but also a key metabolite in living organisms, where it plays an essential role in such fundamental biological processes as respiration and photosynthesis. Due to its ability to exist in equilibrium with carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-, IUPAC's recommended term is *hydrogen carbonate,* but in this review we use its traditional and well-known term *'bicarbonate'*) (see Fig. 1); CO_2 produced by cells during aerobic metabolism of glucose and fats provides the acid (H^+ and CO_2) and base (HCO_3^-) components for the so-called *bicarbonate buffering system*. This buffering system maintains both intracellular and extracellular pH. The interconversion of inorganic carbon, on the other hand, allows rapid transport of its species ($CO_2/HCO_3^-/CO_3^{2-}$) in all cells. While HCO_3^- is poorly soluble in biological membranes, CO_2 can freely diffuse in and out of the cell. Therefore, $HCO_3^- \rightarrow (H_2CO_3) \rightarrow CO_2$ interconversion facilitates the transport of inorganic carbon in the form of CO_2 into intracellular space, while the reversed conversion ($CO_2 \rightarrow (H_2CO_3) \rightarrow HCO_3^-$) provides trapping of the CO_2 within the cell in the form of HCO_3^-. Although the reversible hydration of CO_2 and dehydration of HCO_3^- occurs spontaneously, even in the absence of catalysts, most — if not all — organisms have zinc-containing *carbonic anhydrases* (CAs) that catalyze this ubiquitous conversion. By speeding up these reactions (k_{cat} can reach ~ 1×10^6 s^{-1}), CAs play an essential role in a wide range of biochemical and physiological processes [1].

All photosynthetic organisms need atmospheric CO_2 to store harvested energy from sunlight in the form of energy-rich carbohydrates. However, in the oxygenic photosynthesizers (cyanobacteria, algae and higher plants), CO_2 is not only required as the terminal electron acceptor to synthesize carbohydrates, but also for the regulation of photosynthetic electron transport in Photosystem II (PSII), the enzyme responsible for light-induced primary charge separation and subsequent water oxidation [2]. The latter is known as *the 'bicarbonate effect'*. This review summarizes historical discoveries related to the 'bicarbonate effect' and outlines our current state of knowledge about the location and role of HCO_3^- in PSII.

1.2. The 'bicarbonate effect' and Otto Heinrich Warburg

Despite the fact that the reduction of CO_2 to carbohydrates within the Calvin-Benson cycle is driven by the products of the light reactions of photosynthesis, ATP and NADPH, it does not directly require light, and thus, belongs to the photosynthetic light-independent ('dark') reactions [3]. Until the discovery (1958) of the 'bicarbonate effect' on the light-dependent electron flow by Otto Warburg and Günter Krippahl, CO_2 was assumed to be involved only in the 'dark' and not in the 'light' reactions [4]. Warburg and Krippahl found that the high rates of the Hill reaction (the reaction which allows the study of electron flow in isolated broken chloroplasts illuminated in the presence of an artificial electron acceptor *via* the measurements of O_2 production) required the presence of CO_2 in the gas phase above the sample suspension. Specifically, it was shown that the O_2 evolution rate measured in grana isolated from kohlrabi leaves in the presence of quinone (as electron acceptor) was significantly higher when argon atmosphere above the sample contained 1.4% CO_2 (v/v) (see Fig. 2). In spite of the earlier difficulties with its reproducibility, many research groups confirmed this phenomenon (outlined in Section 3.1). Later on, Alan Stemler and Govindjee [5] significantly improved reproducibility of the bicarbonate effect by developing a reliable method of CO_2/HCO_3^- depletion.

Otto Warburg believed that the observed phenomenon provides evidence for his *'photolyte theory'*, in which O_2 originates from the splitting of *'activated CO_2'*, not from water. In 1964, he noted "As was expected, no proof of water photolysis survived the discovery of 'active CO_2'" [6]. Despite this mistaken interpretation, the finding made by Warburg and Krippahl was fundamental to subsequent research of the 'bicarbonate effect' on light-induced electron transport during photosynthesis. Their discovery initiated long-term debates about possible action site(s) and role(s) of inorganic carbon on photosynthetic O_2 production. Thus, intensive studies by many laboratories explored the possibility that HCO_3^- (CO_3^{2-}) (and not CO_2) is required for both PSII electron transport efficiency and for the photo-assembly of the inorganic core (the Mn_4CaO_5 cluster) of the O_2-evolving complex (OEC) of PSII (see Sections 2 and 3; for previous historical overviews, see [7-14]).

1.3. Photosystem II and the sites of the 'bicarbonate effect'

PSII is a large multi-component pigment-protein complex, which is incorporated into the thylakoid membrane of all oxygenic photosynthetic organisms (for reviews on PSII, see [2, 15]). Fig. 3 shows a schematic view of PSII in higher plants and green algae and its important redox cofactors, which are thought to be the same as in cyanobacteria (for further details on the cyanobacterial PSII structure, see [16] and [17]). PSII acts as a water: plastoquinone oxidoreductase, catalyzing the following reaction:

$$2\ H_2O + PQ + 4\ H^+_{stroma} \xrightarrow{4\ h\nu} O_2 + 2\ PQH_2 + 4\ H^+_{lumen} \qquad (1)$$

Thus, the light-induced charge separation between the reaction center (RC) chlorophyll (Chl) molecules in the D1 protein (Chl_{D1} and P680) and pheophytin ($Pheo_{D1}$), and the formation of the stabilized radical ion pair $P680^{\bullet+}Pheo^{\bullet-}$ (for reviews, see [18, 19]), lead to two reactions: (1) water splitting (oxidation) to O_2, protons and electrons on the lumenal side of PSII, the so-called *electron donor side* of PSII with $P680^{\bullet+}$ as the driving force and, (2) the reduction of plastoquinone (PQ) to plastoquinol (PQH_2) on the stromal, *the electron acceptor side*, of PSII with proton uptake and $Q_A^{\bullet-}$ acting as the reductant.

Extensive data show that HCO_3^- ions, under appropriate experimental conditions, have effects on both the acceptor side and the donor side reactions of PSII (Fig. 3). The focus of this review is the bicarbonate effects related to the electron acceptor side, where HCO_3^- is known to bind (see Fig. 3 and Section 3.2 for the current model) and to play an important role in facilitating the reduction of Q_B, and in protonation reactions near the Q_B site. We, however, will briefly discuss here, for completeness, the possible roles of HCO_3^- on the 'donor side' reactions of PSII. For historical surveys on the discoveries of the HCO_3^- effect on the PSII donor side, see several references [9, 10, 14, 20-22].

2. Bicarbonate and the donor side of Photosystem II

The role of HCO_3^- on the donor side reactions of PSII has been extensively studied by many researchers, but mainly in the laboratories of Alan Stemler (University of California, USA) and Vyacheslav Klimov (Institute of Basic Biological Problems, Russia), as mentioned earlier.

In the early 1970s, Stemler with co-workers [5, 23] were the first to propose the water-oxidizing side of PSII as a possible site for the HCO_3^- effect (Fig. 4). However, in 1975, Thomas Wydrzynski and Govindjee obtained evidence for the participation of HCO_3^- ions in the electron transfer kinetics on the acceptor side of PSII [24] (Fig. 5). This discovery was supported by numerous subsequent experiments (see Section 3), and later on, the non-heme iron (NHI) between Q_A and Q_B was shown to play an essential role in HCO_3^- binding [12, 25]. On the other hand, some (among them Helmut Metzner, Werner Kreutz, and Alan Stemler) believed that HCO_3^- may act as a substrate or a chemical intermediate in photosynthetic O_2 evolution, possibly coupled with CA activity [26-28]. Thus, Stemler and collaborators continued to investigate the possible involvement of HCO_3^- ions in the mechanism of O_2 evolution on the oxidizing side of PSII (reviewed in [9, 22]). Stemler's reports, as well as reports of some others [29, 30] indicated that HCO_3^- may affect both the electron acceptor and donor sides of PSII. Undoubtedly, however, the discovery of the 'acceptor-side' effect inadvertently affected the search for specific effects of HCO_3^- on the donor side, and inevitably led to a controversy on the interpretation of the 'bicarbonate effects'.

Since the mid 1990s, the idea for an additional role of HCO_3^- on the electron donor side of PSII was revived by a series of experiments performed in the laboratory of Vyacheslav Klimov. The studies by Klimov and collaborators indicated that HCO_3^- ions are required for (1) the efficient photo-induced assembly of the Mn_4CaO_5 cluster capable of water splitting, (2) the stability of the OEC, and (3) the protection of the donor side of PSII against photoinhibition and thermoinactivation (reviewed in [10, 14]). Other groups (see, for instance, [31-35]) also obtained indication for the requirement of HCO_3^- on the water-splitting side of PSII. However, the binding site(s) and the role(s) of HCO_3^- ions in the water-splitting reaction of PSII remain unclear (and, therefore, appear 'questionable' to the authors; see Fig. 3). The following main proposals for the involvement of HCO_3^- in the events on the water-oxidizing side of PSII have been considered:

(i) *Exchangeable* HCO_3^- *is an intermediate substrate for photosynthetic water oxidation; water is delivered to the* Mn_4CaO_5 *cluster in the form of* HCO_3^- *(or peroxidicarbonic acid;* $H_2C_2O_6$*).* Initially proposed by Helmut Metzner [27] as an alternative to Warburg's 'photolyte theory' (mentioned above) and later elaborated by Alan Stemler, [26] and by Paul Castelfranco with co-authors [36], this hypothesis has become obsolete, in our opinion, because of various studies using isotope ratio mass spectrometry (MS) in combination with ^{18}O-labeling of H_2O and HCO_3^- [37-41], UV spectrophotometry under high backpressure of CO_2 [38], and light-induced FT-IR difference spectroscopy [42].

(ii) *Non-exchangeable, tightly bound* HCO_3^- *being a structural part of the* Mn_4CaO_5 *cluster may alter the redox properties of the Mn cations, and thus, is required for the functionality and stability of the assembled OEC.* The studies carried out by Klimov and collaborators showed stabilizing and protective effects of HCO_3^- on the donor (water-oxidizing) side of PSII (see, e.g., references [43-50]). One of the interpretations of the observed effects was the idea that HCO_3^- may function as a ligand to the Mn_4CaO_5 cluster or an integral cofactor of the OEC [10]. In addition, in the PSII crystal structure by Ferreira et al. [51] at a resolution of 3.5 Å, HCO_3^- (or CO_3^{2-}) anion was tentatively included as a ligand bridging Mn and Ca ions within the OEC. However, the latter could not be supported by the most recent X-ray crystallography studies of PSII at higher resolutions [16, 17, 52]. At the same time, all these crystallographic studies clearly displayed HCO_3^- as a ligand of the NHI between Q_A and Q_B (for further details, see Section 3.2). Earlier sensitive differential infrared gas analyzer and MS measurements [7, 53] also clearly showed only ~1 HCO_3^-/CO_2 molecule bound per PSII RC (see Section 3.1.2.3). Further, a recent re-examination of the structural coupling of HCO_3^- to the OEC by FT-IR spectroscopy provided no indication for any HCO_3^--bands from the OEC during the S-state transitions [42]. This is also consistent with the results obtained by flash-induced O_2 evolution pattern (FIOP) studies, where the redox potentials of the S states of the OEC were found to be unaffected by HCO_3^- depletion *via* washing with CO_2/HCO_3^--free buffer [54]. Moreover, evidence for the absence of tightly bound HCO_3^- in the first coordination sphere of the Mn_4CaO_5 cluster was obtained by isotope ratio MS [41] (for details, see Section 3.1.3.3) and GC-MS [55] studies. In addition, HCO_3^-, as a structural part of the OEC has not been supported by the computational models based on density functional theory (DFT) and quantum mechanics/molecular mechanics (QM/MM) studies [56, 57]. It is, therefore, very unlikely that HCO_3^- is a ligand or strongly coupled cofactor to the Mn_4CaO_5 cluster in its assembled state.

(iii) *Acting as a transient ligand to Mn ions, HCO_3^- is a native cofactor in the photo-assembly (photo-activation) process of the Mn_4CaO_5 cluster that assembles in the OEC-depleted PSII centers that are free of inorganic cofactors, but HCO_3^- is not part of the assembled cluster.* This suggestion is based on the results obtained by Klimov and co-workers [10] demonstrating a pronounced stimulating effect of HCO_3^- ions on the electron donation from exogenous Mn^{2+} ions to Mn-depleted PSII and the photo-induced reconstitution of the functional OEC [43-45, 48, 58]. Further experiments in collaboration with the group of Charles Dismukes provided evidence for the requirement of HCO_3^- (CO_3^{2-}) for *in vitro* light-driven assembly of the Mn_4CaO_5 cluster (for details, see [31, 59]; for reviews on the photo-assembly of the OEC, see [60, 61]). Although electrochemical characterizations of Mn-HCO_3^- complexes [62-64] as well as electron paramagnetic resonance/electron spin echo envelope modulation (EPR/ESEEM) spectroscopy

studies of assembly intermediates [65, 66] strongly support this idea, there is no experimental data demonstrating a HCO_3^- requirement for the assembly process *in vivo*.

(iv) *HCO_3^- indirectly stabilizes the OEC by binding to extrinsic proteins or some other protein components of PSII in the vicinity of the Mn_4CaO_5 cluster.* Pobeguts et al. [67] demonstrated a protective effect of HCO_3^- against extraction of the extrinsic proteins (especially PsbO, − the Mn-stabilizing protein) of the OEC after treatment of pea PSII membrane fragments with urea. Moreover, the specific high-affinity binding of HCO_3^- (or CO_2) to the PsbO protein has been proposed based on the recent observations of HCO_3^--dependent re-arrangements in the PsbO protein [68]. However, as mentioned above, no HCO_3^- was detected bound on the protein components belonging to the donor side of PSII of thermophilic cyanobacteria by Umena et al. [17] in their recent crystal structure at 1.9 Å resolution. Nevertheless, since significant differences are known to exist between proteins of cyanobacteria and plants (reviewed in [69, 70]), the possibility of HCO_3^- binding to protein components in higher plants needs to be addressed by future experiments.

(v) *Mobile, exchangeable HCO_3^- is involved in proton removal during photosynthetic water oxidation; it may work coupled with the PSII-donor-side-associated CA.* Deprotonation reactions and removal of protons away from the OEC are thought to have significant impact on the thermodynamics of water splitting [71]. Ananyev et al. [34] proposed that HCO_3^- may play an indirect role in water splitting as a proton transfer mediator and recent results support this proposal [32, 35]. In fact, such an interpretation of the bicarbonate effect on the water-oxidizing side of PSII may explain a large body of existing data, especially in connection with the PSII-associated CA activity, which was experimentally shown in many studies [72-78]. Stemler, based on circumstantial evidence, was the first to suggest that a thylakoid CA might be involved in the 'donor-side' effects of HCO_3^- [74, 79-81]. Experiments by Shutova et al. [32] show that in *Chlamydomonas (C.) reinhardtii*, both HCO_3^- and Cah3 (the CA protein in *C. reinhardtii*

associated with the PSII donor side) have specific ‘donor-side’ effects on proton release steps, but not on electron transfer. Moreover, there are also some indications for a similar role of CA and HCO_3^- in higher plans, although both CA and HCO_3^- requirements were found to be lower than that observed in *C. reinhardtii* [82]. Shutova et al. [32] suggested that a CA/HCO_3^- system in *C. reinhardtii* may facilitate proton removal away from the OEC during water splitting by accelerating interconversion between HCO_3^- and CO_2 (see Fig. 1). Indeed, if the lumenal “working” pH under illumination is 5.4-5.7, as shown recently [83, 84], one can assume that due to the strong deficit of HCO_3^- species at this pH range, the presence of CA activity is ‘naturally’ required for the fast production of these species from CO_2.

We note here, that to our knowledge, most of the above bicarbonate-related investigations of cyanobacteria, algae, and higher plants, except for a few with intact alga *Chlamydobotrys stellata* [29] and the hypercarbonate-requiring cyanobacterium *Arthrospira maxima* [85, 86] have been limited to *in vitro* studies of isolated thylakoids, PSII membrane fragments and PSII particles. Therefore, we emphasize that the effect and the function of HCO_3^- on the donor side of assembled PSII may be different (if any) when the protein environment is intact as to when it is disrupted, e.g., as a result of sample preparation. Thus, further research is needed to study this option and to elucidate the role of HCO_3^- on the water-oxidizing side of PSII.

3. Bicarbonate and the acceptor side of Photosystem II

In contrast to what little is known regarding the effect of bicarbonate on the donor side of PSII, we know a great deal about the role of HCO_3^- on the acceptor-side of PSII – and its binding is obvious in the high resolution structures of cyanobacterial PSII RCs. Therefore, the remaining historical perspective in this review will focus on the research efforts related to the discoveries of the bicarbonate effect on the electron flow within PSII, and the HCO_3^--dependent regulation of electron transport on the acceptor side. Hence, the current state of our knowledge about the location and the function of HCO_3^- is also discussed in detail.

3.1. Time-line of discoveries on HCO_3^- in the electron flow of Photosystem II

3.1.1. The Early Work: From Otto Warburg to Norman Good

- In **1948**, Boyle [87] had observed that O_2 evolution by ground-up spinach leaves, when *p*-benzoquinone was added, was absent when KOH was included (to absorb CO_2) in the center well of a manometer vessel; thus, Boyle concluded that CO_2 was necessary for the benzoquinone Hill reaction. Although the conclusion was confirmed by Warburg and Krippahl in 1960 [88], Boyle's results were artifacts as suggested by Warburg and Krippahl (1958) [4] and, as shown, in 1961, by Abeles et al. [89]: benzoquinone in the main vessel distilled into the KOH-soaked filter paper in the center well and the mixture consumed O_2 balancing O_2 evolution from the broken leaves. The discovery of the 'bicarbonate effect' by Warburg and Krippahl has already been mentioned in Section 1.2.
- In **1961**, Abeles et al. [89] confirmed Warburg and Krippahl's results in kohlrabi chloroplasts, i.e., requirement of CO_2 for the Hill reaction. Warburg's idea that O_2 arose from CO_2 (see Section 1.2) had to be tested. For this purpose, Abeles et al. used MS that distinguishes O_2 evolution and metabolism of CO_2. They observed changes only in O_2 release and none in CO_2 metabolism. Thus, Warburg's idea was not supported. However, Abeles and co-workers could not reproduce this effect in sugar beet chloroplasts leading them to conclude that the effect was not universal.
- During **1960-1962**, Stern and Vennesland [90, 91] observed that the ferricyanide-supported Hill reaction, in spinach and kohlrabi chloroplasts suspended in buffered media, declined much faster, with time, without than with CO_2 present. Addition of CO_2 restored Hill activity. Further, in 1963,

Vennesland, who was still supporting Warburg's point of view, reported stimulation of the Hill reaction with different electron acceptors, using thylakoids from various plant sources [92].

- In **1962**, Izawa [93] introduced the use of CA to the reaction medium, while CO_2 was being removed; this hastened the time of CO_2–depletion and gave much more reliable results; Izawa found larger effects in broken than in intact chloroplasts.
- In **1963**, Heise and Gaffron [94] reported decreases in O_2 evolution, during the Hill reaction with *p*-benzoquinone in the cyanobacterium *Anacystis nidulans* (*Synechococcus elongatus* strain PCC 7942) and in the green alga *Scenedesmus obliquus* (strain D3) in the absence of CO_2. However, these authors suggested that this effect is not an important one since many different metabolic reactions have been shown to be dependent on traces of CO_2.
- During **1963-1965**, Good [95, 96] discovered that CO_2 dependence of the Hill reaction, in pea chloroplasts, was highly influenced by the addition of anions, particularly of formate and acetate; none of the anions used could act as bicarbonate; thus, bicarbonate was considered to have a specific stimulatory effect in electron transport during the Hill reaction; uncouplers of phosphorylation had no effect on electron transport in CO_2-depleted chloroplasts.
- During **1964** and **1965**, Punnett and Iyer [97], Punnett [98] and Batra and Jagendorf [99] discovered that in addition to the effects of CO_2/HCO_3^- on electron transport, an additional, although a different effect, exists on photophosphorylation. In their 1978 review on the bicarbonate effect, Govindjee and Van Rensen [11] have called this separate effect, the "*Punnett Effect*"; however, it will not be discussed further in this review since CO_2 was not a requirement for phosphorylation, whereas it is a requirement for electron transport.
- In **1967**, West and Hill [100] confirmed the existence of the stimulatory role of CO_2 in both dichlorophenol indophenol (DCPIP) and ferricyanide Hill reactions in pea chloroplasts, and as Izawa had stated, the effect was larger in broken, than in intact, chloroplasts.

3.1.2. Work at the University of Illinois at Urbana-Champaign (UIUC): From Alan Stemler to Jin Xiong and collaborations with other groups

Most of the research up to this point was aimed to see if there was an effect of CO_2 on the Hill reaction, i.e., electron flow from water to $NADP^+$ (see a review [11]). There were, in general, considerable variations in the magnitude of the effects; further, the conditions producing them showed considerable differences. Govindjee, one of the authors of this perspective, presented a lecture to a graduate level course in late 1960s or early 1970s, where he talked about this effect emphasizing the *out-of-this-world* ideas of Otto H. Warburg — that this effect implies that O_2 comes from CO_2. To the surprise of Govindjee, one of his own doctoral students in the class wanted to pursue this as his PhD thesis project. Govindjee attempted to discourage such an undertaking as it was very risky, but then the student Stemler persisted. The rest is history. We present below a time line of research from 1973 to 1998 in Govindjee's laboratory in this area of research (also see a different perspective in Stemler's reviews [9, 21]). We define the 'bicarbonate effect' as follows: addition of bicarbonate to CO_2/HCO_3^--depleted samples restores fully the electron transport to that without the depletion process.

3.1.2.1. 1970s: Research at the UIUC and collaboration with labs in Berkeley, Leiden and Berlin

- In **1973**, Stemler and Govindjee [5] worked out a procedure (by flushing isolated broken chloroplasts, from maize, with nitrogen in a medium containing a high anion concentration at low pH) to remove bicarbonate; they obtained a large (5-fold) and reproducible effect of bicarbonate on the DCPIP Hill reaction in these samples; they suggested that bicarbonate was bound in darkness and released in light. In view of their experiments with diphenylcarbazide (DPC), they had suggested that the effect was only on the O_2-evolving side of PSII. This suggestion was challenged in 1975 by Wydrzynski and Govindjee [24] (see below).

- In early **1974**, Stemler and Govindjee [101] proceeded to perfect the methods of HCO_3^-–depletion further including the effects of light intensity and differences between the rates of O_2 evolution and ferricyanide reduction, again in broken chloroplasts from maize, suggesting the possible existence of non-O_2-evolving centers, and even an effect of bicarbonate on the rate of photoinactivation. These concepts still remain to be further investigated.
- In **1974**, Stemler and Govindjee [102] reported, working still with broken maize chloroplasts, complex effects of bicarbonate on Chl *a* variable fluorescence induction and delayed light emission, including an initial faster rise of Chl *a* fluorescence (from the minimum "O" level to the intermediate "I" level) in HCO_3^--depleted conditions (in hindsight, a hint of an effect on the electron acceptor side); they suggested that HCO_3^- may stabilize the S_1 state in the dark, and, simply, bicarbonate is of critical importance in the initial photochemical process.
- In collaboration with G.T. Babcock, then at the University of California, Berkeley, Stemler and Govindjee, in **1974** [23] presented the following findings: (1) bicarbonate decreases the probability of so-called "misses" in the system; (2) the turnover time of PSII is increased by CO_2/HCO_3^--depletion since the rate of dark relaxation of the S-states ($S_1' \rightarrow S_2$; $S_2' \rightarrow S_3$) is severely retarded in bicarbonate depleted broken maize chloroplasts (Fig. 4); in our current understanding, this may be either due to effects on the PSII acceptor or the donor or both sides; (3) the final O_2-evolving reaction, after accumulation of four positive charges, is independent of bicarbonate; and (4) bicarbonate has no effect on the dark deactivation of the higher oxidation states, S_2 and S_3.
- In **1975**, Wydrzynski and Govindjee [24], as mentioned above, provided the first evidence that there was a clear effect of bicarbonate on the electron acceptor side of PSII: (1) Absence of bicarbonate led to a faster rise of Chl *a* fluorescence (reflecting reduction of Q_A to Q_A^-) in systems where the O_2-

evolving system was blocked (e.g., by Tris-washing) and artificial electron donors (e.g., NH_2OH, $MnCl_2$, hydroquinone and even DPC) were added to replace water; (2) Effect of increasing concentrations of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; diuron), which blocks electron flow from Q_A^- to Q_B, mimics increasing CO_2/HCO_3^-–depletion conditions (Fig. 5). These results leave no doubt about an effect of bicarbonate on the electron acceptor side of PSII. This, of course, does not mean that there is no bicarbonate effect on the donor side of PSII.

- In **1976**, P. Jursinic, also in Govindjee's lab, and in collaboration with Joseph Warden [103] demonstrated a major effect of bicarbonate on the electron acceptor side of PSII by using three separate and independent methods: EPR signal II "very fast", corresponding to tyrosine Z radical, fast Chl *a* fluorescence yield changes; and delayed light emission; although no effects were observed on the electron donor side of PSII, a reversible inactivation of PSII RC activity was observed.
- In **1976**, Govindjee, in collaboration with M.P.J. Pulles, R. Govindjee, H.J. Van Gorkom and L.N.M. Duysens [104], discovered, using spinach chloroplasts, that bicarbonate-depletion inhibits the re-oxidation of the reduced form of the secondary electron acceptor Q_B (Q_B^{2-}) by the PQ pool. Results on the effects of DCMU supported this conclusion. Flash-number dependent measurements on Chl *a* fluorescence yield established that in CO_2/HCO_3^--depleted samples, the "*two-electron gate*", on the electron acceptor side of PSII, was non-functional (Fig. 6).
- In **1977**, Khanna et al. [105] performed the first, so-to-say, *biochemical surgery* of the bicarbonate effect, using artificial electron acceptors (silicomolybdate (SM), oxidized diaminodurene (DAD), and methyl viologen (MV)) and donors (DPC, reduced DAD), acting at specific sites, and the inhibitors (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) and DCMU) of electron flow, also at specific sites (see Fig. 7). Results were clear: (1) there was no bicarbonate effect on Photosystem I (PSI); (2) there was no bicarbonate effect on the water–oxidizing side of PSII; and (3) there was a definite inhibition of electron flow on the PSII acceptor side, in agreement with the work of

Wydrzynski and Govindjee [24].

- In **1977**, in collaboration with U. Siggel and G. Renger, in Berlin, R. Khanna and Govindjee performed absorption spectroscopy to decipher the nature of electron carriers on the PSII acceptor side that were affected by CO_2/HCO_3^--depletion and the reversal after HCO_3^- re-addition [106]. The formation of Q_B^{2-} was reversibly slowed down, by a factor of 10-20 fold, from ~500 μs to ~8 ms. However, a much larger effect of CO_2/HCO_3^--depletion was in the slowing down of the reduction of PQ pool to ~100 ms; this was consistent with the measurements of Govindjee et al. [104], where the fluorescence decay after the 3rd and subsequent flashes was in the range of 150 ms; this was also reflected in the slowed reduction of oxidized P700. This is clearly the major bottleneck produced by bicarbonate depletion.

3.1.2.2. 1980s: Further research at UIUC, and collaboration with other labs in Berlin and in Wako Shi (Japan)

- In view of the fact that reduction of Q_B to PQH_2 requires protonation, it became obvious that bicarbonate must be playing a role through protonation, and, thus, in **1980**, in collaboration with Wolfgang Junge's research group, in Berlin, Khanna et al. [107] measured the effect of HCO_3^--depletion on the proton uptake and release, using pH indicator dyes neutral red (internal space) and bromo-cresol purple (external space); the results of bicarbonate-depletion on protons were remarkable: not only the release of protons into the internal space was dramatically reduced, there was no proton uptake by the PQ pool at the outer side of the membrane (Fig. 8). Whatever be the detailed mechanism, effects on protonation by bicarbonate depletion on PSII were firmly established.
- In **1981**, and in collaboration with the research group of Charles Arntzen, and with Jack Van Rensen, Khanna et al. [108] provided information suggesting that the binding of bicarbonate is on the same protein that binds the herbicide atrazine; further results suggested complete inactivation of a part of

the total number of electron transport chains. These conclusions were based on: (1) a shift in the binding constant of atrazine in bicarbonate-depleted thylakoid membranes indicating decreased affinity of atrazine; (2) trypsin treatment, which modifies PSII at the level of Q_B, strongly diminished stimulation by bicarbonate addition to HCO_3^--depleted thylakoids. These conclusions were confirmed by measurements on atrazine-resistant plants (Fig. 9).

- In **1982**, in collaboration with Van Rensen, Vermaas et al. [109] used the herbicide ioxynil, which is different from atrazine used earlier by Khanna et al. [108]; inhibition of electron transport by ioxynil increased at decreasing bicarbonate levels (Fig. 10). An interesting conclusion of this study was that the binding sites of herbicide and bicarbonate, although similar, are not identical.
- In **1984**, Blubaugh and Govindjee [30] came to the conclusion that bicarbonate has 2 binding sites: (1) a high affinity binding site close to where DCMU binds; this binding is inhibited by light; and (2) a low affinity binding site, which requires light, and is where bathocuproine may bind, and, thus, this could be the one effect on the donor side of PSII. These results and conclusions need further investigations. Considering the high resolution structure of PSII [17] where only one bicarbonate site has been seen, all experiments dealing with two bicarbonate binding sites need to be re-examined and proven by additional experiments using newer methodologies.
- In **1984**, Eaton-Rye and Govindjee [110] extended the conclusions of Khanna et al. [105], using MV as electron acceptor, and provided additional evidence that there was no effect of bicarbonate in PSI, and that there was a specific effect on the PSII electron acceptor side (reduction of PQ) that was not dependent on the use of formate to remove bicarbonate. They suggested "the observed large slow component in HCO_3^--depleted samples results from an altered equilibrium of Q_A^- with PQ and/or PQH_2 at the Q_B binding site". This conclusion was consistent with their hypothesis that removal of HCO_3^- results in a retardation of the PQ/PQH_2 exchange reactions of the two-electron gate. Further, it has been suggested that this may be due to changes in the association constants for one or more of

the PQ/PQH_2 species and/or by affecting the protonation reactions of the partially reduced plastosemiquinone anion or the doubly reduced plastoquinol; it was only after the first full turnover of the two-electron gate that the full effect of HCO_3^- depletion could be observed. This also explained the observation of Govindjee et al. [104] and Robinson et al. [111] that the decay of Chl *a* fluorescence after the 1st flash is less inhibited than after the 3rd and subsequent actinic flashes, but intermediate after the 2nd flash.

- In **1984**, Govindjee et al. [112], working in the laboratory of Y. Inoue (Wako Shi) in Japan, confirmed, through thermoluminesence (TL) measurements that the bicarbonate depletion affected PSII on the electron acceptor side, in the Q_AQ_B region. They discovered (1) a 6-10°C shift, to a higher temperature, in the $S_2Q_B^-$ TL band; (2) a reduction in TL intensity upon prolonged depletion of bicarbonate; and (3) elimination, after the first few flashes, of the characteristic period four oscillations in TL intensity as a function of the flash number. On the other hand, addition of DCMU produced the same $S_2Q_A^-$ TL band, at about +20^{o}C in both depleted and reconstituted samples. These results suggest (1) the initial effect of CO_2/HCO_3^--depletion is to increase the activation energy for $S_2(S_3)Q_B^-$ recombination; (2) with further depletion, the incidence of this recombination decreases and the cycling of the S_2 Q_B^- and $S_3Q_B^-$ recombination is inhibited through effects at the Q_B apo-protein. These bicarbonate depletion effects were fully reversible if HCO_3^- was added to HCO_3^--depleted samples (i.e., reconstituted samples). A conformational change of the PSII complex in the region of the Q_B apo-protein was suggested to be responsible for these effects.
- The CO_2 concentration in water solutions ($[CO_{2(aq)}]$) is a function of Henry's Law of solubility and the partial pressure of CO_2 (g) in the air above the water (see Fig. 1). Concentrations of other inorganic carbon species, i.e., HCO_3^- and CO_3^{2-}, vary with pH, and therefore, the ratio $[HCO_3^-]/[CO_{2(aq)}]$ is pH dependent (for details, see [113]). The total concentration of dissolved inorganic carbon increases at the pH range between 6 and 9 due to an increase in HCO_3^- species. In **1986**, Blubaugh and Govindjee [114], taking advantage of the pH dependence of the ratio $[HCO_3^-]/[CO_2]$ at

equilibrium to vary effectively the concentration of one species while holding the other species constant, discovered that the Hill reaction was stimulated in direct proportion with the equilibrium [HCO_3^-], but was independent of the equilibrium [CO_2] (Fig. 11). Thus, they suggested that HCO_3^- is the species, which binds to the effector site, while CO_2 is the diffusing species [115].

- In **1988**, Blubaugh and Govindjee [116], using kinetic analysis of rates of electron flow *versus* [HCO_3^-], came to the conclusion that there are two high affinity bicarbonate binding sites, apparently with cooperative binding. We now ask where is the second bicarbonate binding site, if it really exists? On the PSII electron donor side? Or at another site on the electron acceptor side? As mentioned above, since in a recent high-resolution PSII structure there is no indication for two HCO_3^- molecules [17] (also see Section 3.2) the two binding site concept needs to be re-examined with new experimental approaches. Another conclusion was that bicarbonate is an essential activator for PSII and that complete removal of bicarbonate would result in zero electron transport activity [116].
- In **1988**, Eaton-Rye and Govindjee [117, 118] provided a detailed study of flash number dependent analysis of Chl *a* fluorescence decay in spinach thylakoids at different pH values. This study provided several important conclusions. The concept that bicarbonate was involved in protonation was fully supported: A model of bicarbonate acting as a proton donor to the protein dissociable group believed to participate in the protonation of reduced Q_B was discussed, as well as the possibility of bicarbonate being a ligand to the NHI in the Q_A-Fe-Q_B complex of the PSII RC. In addition, in **1988**, (1) Cao and Govindjee [119] reported bicarbonate effect in a cyanobacterium *Synechocystis* sp. PCC 6803; and (2) in collaboration with Govindjee, Garab and collaborators [120] provided evidence through TL measurements that CO_2 does affect charge accumulation in intact leaves.
- In **1989**, (1) in collaboration with the lab of Tony Crofts, Govindjee et al. [121], using fast fluorescence changes, failed to observe any significant effect of bicarbonate on electron donation

from tyrosine Z (Y_Z) to P680 or in the formation of $P680Q_A^-$; and (2) in collaboration with the lab of Colin Wraight [122], a total absence of CO_2/HCO_3^--depletion effect was observed between the quinones both in chromatophores and RCs in the purple bacterium *Rhodobacter (R.) sphaeroides.* This was followed, in **1992**, by the work of X. Wang (in Colin Wraight's Lab at Urbana, IL) and J. Cao (in Govindjee's lab) who, in collaboration with Dieter Oesterhelt's lab in Munich, Germany [123] asked if bicarbonate in PSII is equivalent of Glu (M234 in *R. sphaeroides*) in bacterial RCs in binding to the NHI? Michel and Deisenhofer [124] had earlier suggested this notion. None of the mutants of M-234, where Glu was changed to Val, Gln or Gly, showed any difference in the HCO_3^--reversible formate effect, confirming the absence of bicarbonate effect in these anoxygenic photosynthetic bacteria.

3.1.2.3. 1990s: Continued research at UIUC, and collaboration with other labs in the USA and in labs around the World (Canada, China, Israel, Finland, Switzerland, France, Germany, and The Netherlands)

- Following the lead of Khanna et al. [108] and Vermaas et al. [109] that had suggested an overlap of binding sites of bicarbonate and herbicides in higher plants, Govindjee, working in collaboration with C. Vernotte, B. Peteri, C. Astier and A.L. Etienne, found, in **1990** [125] that the herbicide-resistant mutants of the cyanobacterium *Synechocystis* sp. PCC 6714, that are altered in specific amino acids in their Dl protein, show differential sensitivity to formate treatment. Yield of O_2 in a sequence of flashes, Chl *a* fluorescence transients and Chl *a* fluorescence yield decay after a flash revealed that the resistance of cells to formate treatment was in the following (highest to lowest) order: [double D1-mutant] A251V/F211S > [single D1-mutant] F211S > wild type > [single D1-mutant] S264A. These results established the involvement of the D1 protein in bicarbonate/formate binding, but gave no further clue to the precise site of binding. From the PSII crystal structure [17], these residues are rather close to Q_B; changes in these residues may perturb the proper binding of Q_B, giving rise to indirect effects on the binding of bicarbonate/formate.

- In **1991**, using membrane-inlet mass spectrometry (MIMS) and a infrared gas analyzer, Govindjee in collaboration with H.G. Weger, D.H. Turpin, J.J.S. van Rensen, O.J. devos and J.F.H. Snel, [53] showed that formate replaces HCO_3^- from its binding site in PSII (see Fig. 12 and legend for experimental details). Addition of 100 mM formate to spinach thylakoids released ~0.4 HCO_3^-/CO_2 to 1.3 HCO_3^-/CO_2, confirming the earlier idea [12, 125] that the bicarbonate effect occurs through the binding of HCO_3^- to PSII, and that the addition of formate removes HCO_3^-/CO_2 from its binding site, leading to inhibition of electron flow. This did not support the experiments and conclusions of Alan Stemler [126]. It appears that about 1 HCO_3^- (at pH 6.5) is released by formate addition. Further, in 1995, Oscar et al. [127] established the "bound-bicarbonate" rather than the "inhibitory anion or the empty site" hypothesis of Jursinic and Stemler [128] by showing CO_2 release under their experimental conditions.
- Further evidence that the D1 protein was involved in the HCO_3^- effect on PSII was obtained, in **1991**, by Govindjee et al. [129], using a D1-L275F strain and several other mutants o*f C. reinhardtii*, in collaboration with laboratories at University of Geneva, Switzerland. The L275F mutant failed to show the HCO_3^--reversible formate effect suggesting to the authors that a significant change in formate (bicarbonate) binding had occurred in helix V of the D1 protein near His involved in NHI binding. Further, with the exception of the S264A mutant, which is considerably more sensitive to formate than the wild type, five other different [V219I, A25IV, F255Y, G256D and cell-wall deficient CW-15] mutants displayed a relatively similar response to formate as wild type. Absence of a formate effect on a PSII-lacking mutant seemed to confirm the sole involvement of PSII in the 'bicarbonate effect'. These results suggested that specific areas of the D1-protein are more important than the others in formate/bicarbonate binding, but they did not give precise clues. Lack of effect may not only be due to geometric organization of the structure, but may also be due to a replacement

with similar residues. The search continued.

- In **1991**, Xu and Govindjee [130], in collaboration with the laboratory of Tony Crofts, presented a detailed kinetic investigation on spinach thylakoids, as well as a model of HCO_3^--reversible formate/formic acid effect. In agreement with earlier reports [104, 118], electron flow from Q_A^- to Q_B^- was shown to be slowed down, and the notion that CO_2/HCO_3^--depletion leads to a loss of protonation necessary for stabilization of Q_B^- became the dominant mechanism. However, their conclusion that it is formic acid, not formate, which binds to the acceptor side of PSII has not been pursued since then, and it remains to be further investigated and deserves additional studies.
- In their detailed review in **1988**, Blubaugh and Govindjee [12] had presented models for HCO_3^- action in PSII, and had also suggested that positively charged Arg residues may be involved in bicarbonate binding. In order to test this idea, J. Cao, W. Vermaas and Govindjee [131], in **1991**, made D2-R233Q and D2-R251S mutants in the cyanobacterium *Synechocystis* sp. PCC 6803, and, based on both O_2 evolution and Chl *a* fluorescence measurements, suggested that these two Arg residues must be somehow involved in bicarbonate binding in PSII. In the current high-resolution PSII structure [17], these two residues are located on the stromal surface 15-16 Å away from bicarbonate, and thus their effect may be indirect, through effects on the hydrogen-bonding network linking the bicarbonate to the stromal surface.
- In **1992**, in collaboration with Klaus Pfister and Reto Strasser's research group, Govindjee et al. [132] extended the earlier work on several herbicide resistant D1-*C. reinhardtii* mutants [129] and concluded that D1-S264, but not D1-L275, D1-F255 and D1-V219, plays an important role in the functioning of HCO_3^- and PQ in PSII; the role of D1-G256 could not be determined in this study. (See also Strasser et al. [133] for the role of D1-S264 and the absence of the role of D1-L275.) The high-resolution structure of PSII [17] now shows that D1-S264 is hydrogen-bonded to Q_B (see Section 3.2).

- The role of various D1 amino acids in the HCO_3^- effect, this time, by using herbicide-resistant mutants of the cyanobacterium *Synechococcus* sp. PCC 7942, was examined by Cao and Xiong, in **1992**, in collaboration with Hirschberg and Ohad, in Israel [134]. Interestingly, the hierarchy of the equilibrium dissociation constant for bicarbonate (highest to lowest) was D1-F255L/S264A > D1-F255Y/S264A ~ D1-S264 ~ D1-F255Y > Wild type, establishing the importance of D1-S264 and D-F255 in the HCO_3^- binding niche directly or indirectly. Again, a role of bicarbonate in protonation and stabilization of Q_B^- was emphasized, a recurring concept since the earlier observations [107].
- By **1993**, the following conclusions were made [135]: (1) Formate, azide, nitrite and nitric oxide inhibited electron flow in thylakoids and cells, and these effects were significantly and uniquely reversed by bicarbonate; (2) with formate treatment, a remarkably strong HCO_3^--reversible slowing down of Q_A^- reoxidation after the second and subsequent flashes, but not after the first flash, was observed; (3) a hypothesis was in place suggesting that bicarbonate functions as a proton shuttle stabilizing the binding niche of Q_B^- and stimulating PQH_2 formation (and, perhaps, even its oxidation) in some manner; (4) this effect somehow involves both D1 and D2, directly, or indirectly, particularly the region where herbicides bind, and part of this was based on several mutant studies (e.g., D1-S264A, D1-L275F, D2-R251S, D2-R233Q, D2-R139H, among others); (5) possible involvement of "Fe" in the "Q_A-Fe-Q_B" complex was also implicated; (6) this effect was unique to PSII since electron transport in the "Q_A-Fe-Q_B" complex of both green and purple bacteria (including M-E234G, Q and V mutants) were insensitive to HCO_3^--reversible inhibitors.
- In **1995**, Mäenpää et al. [136] made an interesting observation in *Synechocystis* sp. PCC 6803: HCO_3^--reversible formate effect on Q_AQ_B was several fold less in the CA1 mutant (that had Glu 242, Glu 243, and Glu 244 deleted, and where Gln 241 was changed to His; these changes being in the de-loop of the D1 protein). These results may be related to differences in the accessibility of the anions and/or due to changes in the redox properties of Q_A/Q_A^- in the mutant—perhaps, an indirect effect.

- In **1996**, in collaboration with the research group of Richard Sayre, attempts were made to test the importance of D1-R269 in *C. reinhardtii* [137]; it was difficult to obtain firm conclusions since the used D1-R269G mutant was unable to grow photosynthetically and to evolve O_2: it had many defects. In the current high-resolution PSII structure [17], D1-R269 is hydrogen-bonded to D2-T243, which is probably needed to maintain the proper orientation of D2-Y244 in order for it to be able to hydrogen-bond to the bicarbonate (see Section 3.2).
- In **1996**, Xiong et al. [138] presented a 3-dimensional model of the D1/D2 protein and the cofactors, using the bacterial RCs, and predicted the HCO_3^- binding niche in PSII; it was modeled in the NHI site, providing a bidentate ligand to the iron. In their model, a bicarbonate ion was suggested to be stabilized by D1-R257, was said to donate a proton to Q_B^{2-} through the D1-H252 residue, whereas a water molecule was proposed to donate another proton to Q_B^{2-}; Xiong et al. also proposed a positively charged water channel, near Q_B and the NHI, for transporting water and HCO_3^-. It is now indeed known [17] that D1-H252 is hydrogen-bonded to Q_B through D1-S264, and there are water molecules close to D1-H252 that could serve as proton donor to Q_B (see Section 3.2). However, D1-R257 is at a distance of 8.6 Å from Q_B and further away from the bicarbonate; thus, it cannot be directly involved in this hydrogen-bond network.
- In **1997**, Govindjee et al. [7] presented data on Chl *a* fluorescence yield changes after light flashes 1-6 in spinach thylakoids at pH 6.0; they showed a bicarbonate effect on both the electron donor and electron acceptor sides in the same samples. The donor side effect was shown by a decrease in maximum fluorescence, and the acceptor side effect by a slowing down of the fluorescence decay due to Q_A^- oxidation. Using a sensitive differential infra-red gas analyzer they showed the presence of 0.8—1.25 bicarbonate ions bound per PSII RC in maize and pea thylakoids. These results were in agreement with earlier published data obtained by time-resolved MIMS on spinach thylakoids [53] (Fig. 12). Govindjee et al. [7] suggested that bicarbonate bound to the acceptor side is required for

PSII activity, both on the acceptor and the donor sides in the same experiment and in the same sample; in this hypothesis, conformational changes may need to be invoked.

3.1.2.4. 2000s: New conclusions, collaboration with research group of Tony Crofts

- In **2008**, Rose et al. [139], using both Chl *a* fluorescence, and TL measurements, provided the following conclusions on the D1-R257 mutation (D1-R257E, D1-R257M, and D1-257K): Although the forward rate of electron transfer from Q_A to Q_B was little affected, the two-electron gate on the acceptor side of PSII was thermodynamically perturbed in the R257 mutants; this led to a decrease in the overall electron transfer rate from water to PQ. The effects on equilibrium constants of the two-electron gate are likely due to changes in coulombic fields on changing the net charge in the neighborhood of the Q_B site, suggesting that the electrostatic environment plays an important role in the mechanism of PSII. The bicarbonate-reversible formate effect on the Q_B site had been shown to be on the protonation events at this site [117, 118]. Dramatic differences of the bicarbonate effect on the D1-R257 mutants, observed earlier [140], might thus have a basis in changes in the redox potential and the stability of the Q_B site, observed in this research. It, thus, seems that although D1-R257 is not close to the binding site of HCO_3^- on the NHI, it has a significant effect on the PSII reactions in the Q_B region.
- As the model for the role of HCO_3^- had been evolving, it was generally thought that the first proton for the stabilization of Q_B^- came from D1-H252, and, thus, removal of bicarbonate did not exhibit its major effect on the electron transport from the reduced Q_A to Q_B, but it had a large effect on the electron flow from the reduced Q_A to Q_B^-, and the succeeding reactions; the idea that bicarbonate provides this second proton, becoming carbonate, is the current picture. Carbonate, in turn, picks up a proton from D1-E244, finishing the cycle. The HCO_3^- ions (or water protons) outside the PSII complex provide the missing protons to the Glu (see current model in Section 3.2). The idea of

involvement of D1-H252 in the first protonation was discussed by Petrouleas and Crofts [141], based on the experiments of S. Padden (see [142]; and paper in preparation). We note that depending upon the severity of bicarbonate depletion procedure, an inhibition after the 1st flash is also observed explaining effects on TL band due to $S_2Q_B^-$ recombination (see earlier discussion).

3.1.3. Work around the World related to the site of bicarbonate binding

Research summarized below focuses on the studies related to key observations of bicarbonate binding to the electron acceptor side of PSII, mainly on the Q_A-NHI-Q_B nich and the PQ pool.

3.1.3.1. The 1980s

- In **1984**, Vermaas and Rutherford [143] were among the first ones to focus on the relationship of bicarbonate to the Q_A-NHI-Q_B niche of PSII. They discovered that removal of HCO_3^-/CO_2, in PSII membrane fragments from *Brassica napus*, led to a very large increase in the EPR signal at $g = 1.82$ that is due to the $Q_A^-Fe^{2+}$ complex, and, that this effect was fully reversible when bicarbonate was added back. This result identified bicarbonate to be either located near this complex, or, to play a crucial role in affecting the conformation of the Q_AFe complex.
- In **1987**, Diner and Petrouleas [144] showed reversible decrease in the quadrupole splitting of the NHI Mossbauer spectra, upon bicarbonate depletion. This confirmed the concept of bicarbonate acting on the electron acceptor side of PSII.
- In **1988**, Nugent et al. [145], using EPR measurements on both NHI ($g = 6$) and $Q_A^-Fe^{3+}$ ($g = 1.82$) in PSII particles, from both the thermophilic cyanobacterium *Phormidium laminosum* (Fig. 13A) and *Spinacea oleracea* (spinach) (Fig. 13B), suggested that bicarbonate binds close to the NHI and affects Q_A, Q_B as well as the NHI. Further, they found that the NHI was oxidized only when bicarbonate was present (also see [146]). These results supported the conclusions of Govindjee and

coworkers (see Sections 3.1.2.1 and 3.1.2.2) that bicarbonate plays a central role in providing conditions for efficient electron flow on the acceptor side of PSII [11, 147].

- In **1988**, Michel and Deisenhofer [124] in their perspective in the journal *Biochemistry* wrote "Having in mind the well-known effects of bicarbonate at the electron-accepting site of PSII, we consider bicarbonate as a likely candidate to be the fifth iron ligand in D1 and D2". They suggested that bicarbonate occupies the place of M-E232 of anoxygenic bacterial RC.

3.1.3.2. The 1990s

- In **1990**, Diner and Petrouleas [25], using NO, instead of formate, to remove CO_2/HCO_3^-, showed that g = 4 EPR signal of Fe^{2+}-NO was diminished when bicarbonate was added, favoring the concept that HCO_3^- is a ligand to the NHI.
- In **1991**, Diner et al. [148] presented a detailed overview on the iron-quinone electron acceptor complex of PSII. Here, they reviewed the literature on the bicarbonate effect in PSII and discussed various models for the binding and functioning of bicarbonate at the Q_A-NHI-Q_B complex.
- In **1995**, Hienerwadel and Berthomieu [149] provided the first IR spectroscopy evidence for bicarbonate binding on the acceptor side of PSII, using FT-IR difference spectroscopy, and ^{13}C-labeled HCO_3^-. Binding of bicarbonate to the NHI was strongly supported by this study; it was suggested that bicarbonate is a monodentate ligand of the oxidized iron, but a bidentate ligand of the reduced form of iron, and exhibits hydrogen bonds with the protein.

3.1.3.3. The 2000s

- In **2001**, Berthomieu and Hienerwadel [150] looked for the specific interactions of bicarbonate with the protein; here, they used lactate, glycolate and glyoxylate, instead of formate or NO, to remove inorganic carbon. Further, these authors concluded, from their studies, that one proton is released upon iron oxidation, and suggested that pH dependence of the iron couple may reflect deprotonation of D1-H215, a "putative" iron ligand located at the "Q_B" pocket. (This proton release was suggested to have a different mechanism from that involved in the functioning of bicarbonate.) They concluded that a *'hydrogen network'* exists from the NHI towards the "Q_B" pocket involving bicarbonate and D1-H215 (see current model in Section 3.2).

- In **2008**, in search for proof (or absence of proof) for the binding of HCO_3^- to the electron donor side of PSII, Shevela et al. [41, 151] re-examined and extended the MIMS experiments reported earlier by Govindjee et al. [53] and Stemler [126]. Govindjee et al. [53] had presented clear evidence for the release of CO_2/HCO_3^- induced by formate addition (Fig. 12); however, the binding site for this anion was not specified in this study. Based on the previous experimental data, indicating the binding of HCO_3^- to the NHI at the acceptor side, it was assumed that formate removes HCO_3^- from this binding site. Formate, however, was reported to bind both at the acceptor and donor sides of PSII [152]. It was, therefore, unclear, from which binding side(s) in PSII the released CO_2 had originated in previous study [53]. In the MIMS study of Shevela et al. some experiments were performed with an $H_2{}^{18}O$ enrichment, which allowed the detection of CO_2 isotopologues at $m/z = 46$ ($C^{16}O^{18}O$), and $m/z = 48$ ($C^{18}O_2$). Since the Faraday cups used for the detection of $C^{16}O^{18}O$ and $C^{18}O_2$ were amplified by 10 and 100, respectively, than the one used for the detection of non-labeled CO_2 ($m/z = 44$), the ^{18}O-enrichment greatly increased the sensitivity of the MS instrument (compare signal amplitudes in Fig. 14A, B, and C). The results obtained not only fully confirmed the formate-induced release of CO_2/HCO_3^- reported earlier by Govindjee et al. [53] (Fig. 12), but also clearly demonstrated that the released HCO_3^-/CO_2 originates *only* from the acceptor side, and *not* from the donor side of PSII (for experimental details see Fig. 14 and its legend). We also note here that, in the same year (**2008**), evidence for the binding of HCO_3^- on the electron acceptor side of PSII and the absence of bicarbonate bound to the donor side was presented in a FT-IR spectroscopy study by Aoyama et al. [42] and in a GC-MS study by Ulas et al. [55]. Thus, the focus of action on the mechanism remained on the Q_A-NHI-Q_B complex.

- In **2009**, Cox et al. [153] continued EPR studies on the Q_A-NHI-Q_B complex of PSII, initiated in the 1980s and 1990s (see above), but they added DFT calculations. They looked at the native *g* ~ 1.9 form as well as the *g* ~ 1.84 form, which is the well known signal in purple bacterial RCs (where bicarbonate does not bind, see Section 3.1.2.2) and that is occurring in PSII when they are treated with formate that removes CO_2/HCO_3^{-}. The calculations led Cox et al. to conclude that the doubly charged carbonate ion (CO_3^{2-}) is responsible for the *g* ~ 1.9 form of the semiquinone-iron signal; and carbonate, rather than bicarbonate (HCO_3^{-}), is the ligand to the NHI; the latter is in apparent contradiction to what we believe was the conclusion of Berthomieu and Hienerwadel (see above). It is highly likely that both bicarbonate and carbonate can bind to the NHI depending upon the precise physical and chemical status of the system since carbonate is formed from bicarbonate when the latter would be donating a proton to stabilize Q_B^{2-} (see Section 3.2)
- In **2009**, Takahashi et al. [154] dug deeply into the question of HCO_3^{-} binding at the NHI in PSII using FT-IR, as Berthomieu and Hienerwadel [150] had done, and included DFT calculations as well. Their study included specific ^{13}C-Tyr labeling together with a deuteration effect to provide evidence from Tyr IR modes to indicate Tyr involvement in hydrogen bonding to bicarbonate. The results obtained indicated that a Tyr (either D1-Y246 or D2-Y244; see Section 3.2) side chain in “a hydrogen bond donor-acceptor form” is strongly coupled to the NHI; this was suggested to provide a hydrogen bond to the oxygen of the bicarbonate ligand. Thus, Takahashi et al. were the first to propose that a key “Tyr residue coupled to the NHI may play a key role in the regulatory function of the iron-bicarbonate center by stabilizing the bicarbonate ligand and forming a rigid hydrogen bond network around the NHI.”
- In **2011**, Sedoud et al. [155] provided a thorough study on the effects of formate binding on the EPR of the quinone-NHI electron acceptor complex using light flash experiments and reached the

conclusion that the effect was maximum after the 3rd flash indicating that the major effect of formate treatment (HCO_3^-/CO_2 removal) is on the Q_BH_2 exchange. This conclusion is in agreement with the earlier results of flash number dependence on Chl *a* fluorescence observed by Govindjee et al. [104] and on absorption changes by PQ, as measured by Siggel et al. [106]. However, this does not preclude, at all, the participation of bicarbonate in the protonation of Q_B^{2-}. An integrated model would include both effects although the bottleneck reaction that would control the net electron flow may very well be this exchange reaction that would lead to slower oxidation of PQH_2.

- In **2011**, Chernev et al. [156] investigated the NHI-(bi)carbonate complex using µs-resolution X-ray absorption spectroscopy (XAS) after laser flash excitation of PSII membrane particles. An interpretation of the observed spectral changes revealed that the coordination of bicarbonate at the Fe^{2+} may change from a bidentate to a monodentate ligation (carboxylate shift) after the formation of Q_A^-. Based on the obtained data and DFT calculations as well as on previous XAS experiments showing that no $Fe^{2+} \rightarrow Fe^{3+}$ transition occurs during the electron transfer from Q_A to Q_B in the type II photosynthetic RCs [157], Chernev et al. proposed that a coordination flexibility of the ligand (bicarbonate in PSII and glutamate in bacterial RCs) is essential for the functioning of the NHI-carboxyl complex in the interquinone electron transfer.
- In **2011**, Müh et al. [158] have beautifully reviewed PQ reduction in PSII. They suggest that one water molecule is there in the PSII structure that interacts with D1-H252, and two water molecules bridge D1-E244 and D1-Y246, and these could very well be involved in proton pathways (see Figs. 4A and 6A in [158]). They independently propose, as Govindjee, in collaboration with T. Crofts and S. Padden [142, 159] has suggested that the first proton enters *via* D1-H252 and the second *via* D1-Y246, and that it may involve D1-E244.

3.2. The crystal structure at a resolution 1.9 Å and the current model for bicarbonate function

The crystal structure of PSII was first reported by Zouni et al. in 2001 [160] at a resolution of 3.8 Å from a thermophilic cyanobacterium *Thermosynechococcus (T.) elongatus*. Subsequently, Kamiya and Shen reported, in 2003 [161], the PSII structure from *T. vulcanus* at a 3.7 Å resolution. These structures did not allow the assignment of bicarbonate in PSII, either on the donor, or the acceptor side. The first assignment of bicarbonate was reported by Ferreira et al., in 2004 [51], in their PSII structure from *T. elongatus* at 3.5 Å resolution, in which they assigned two HCO_3^- ions, one at the donor side and the other at the acceptor side. The bicarbonate at the donor side was assigned to be a direct ligand to the Mn_4CaO_5 cluster. The density that was assigned to a putative bicarbonate, however, was not found in the subsequent structures at higher resolutions of 2.9-3.0 Å [52, 162]. In the most recent structure of PSII determined at a resolution of 1.9 Å [17], bicarbonate was also not found at the donor side. Since in this high resolution structure, all of the ligands for the 4 Mn ions and the Ca ion were determined, which showed that each of the Mn has 6 ligands and the Ca ion has 7 ligands, there is no room for the presence of a bicarbonate in the immediate ligand sphere of the Mn_4CaO_5 cluster, at least in the assembled, active PSII complex. It is also highly unlikely that a well-defined HCO_3^- could be missed in an electron density map with a resolution beyond 2.0 Å, as the electron density for the bicarbonate at the acceptor was clearly defined and visible [17]. One can assume that bicarbonate strongly bound to the Mn_4CaO_5 cluster might be lost due to reduction of high-valence Mn ions ($Mn^{III}_2Mn^{IV}_2$) to Mn(II), which is known to take place under X-ray doses used for structure determination by X-ray crystallography [163]. However, in view of recent MS and FT-IR data [41, 42, 55, 151] showing the absence of tightly bound bicarbonate to the Mn_4CaO_5 cluster, this option can be excluded.

Fig. 15A shows the position of the bicarbonate on the acceptor side in a PSII monomer determined at 1.9 Å resolution [17]. While the global position of the bicarbonate could be assigned in the structures with a resolution in the range of 3.0-3.5 Å, its detailed environment including the presence and the positions of water molecules surrounding it has to be determined at a much higher resolution, which is now achieved at 1.9 Å. Based on this structure, the bicarbonate serves as a bidentate ligand to the NHI, which is located just under the surface of the stromal side of the membrane region. This bicarbonate is surrounded by hydrophilic residues and water molecules, indicating that it is in a highly hydrophilic environment.

As we can see from Fig. 15B, there is a very small proteinaceous region from the HCO_3^- toward the stromal solution; thus, protons from the stromal side are expected to have easy access to the site of bicarbonate. In order for an efficient and uni-directional transfer of protons to be able to occur, however, hydrogen-bond networks are expected to be present. In fact, well-defined hydrogen-bond networks have been found linking the bicarbonate to the stromal bulk solution. As shown in Fig. 16, the 3rd oxygen in the bicarbonate that was not ligated to the NHI is hydrogen-bonded to a water molecule (W1138A in the 1.9 Å structure, PDB code 3ARC). This water molecule has four hydrogen bonds with its neighboring groups, among which, two are Tyr and Ser residues of the D1 protein (D1-Y246 and D1-S268), and the 3rd one is another water molecule (W675A). This 2nd water molecule (W675A) extends the hydrogen-bond network to the stromal surface through another water molecule W2195D. A plausible hypothesis is: After reduction of Q_B by the reduced Q_A, protons could be easily taken in from the stromal bulk solution through this hydrogen-bond network, and transferred to the site of bicarbonate, which may be further transferred to the reduced Q_B through D1-H272 and D1-H215.

Both the 1st (W1138A) and the 2nd (W675A) water molecules, which are hydrogen-bonded to the bicarbonate, have a tetragonic configuration, bearing 4 hydrogen bonds with their neighboring molecules. The amino acid residues surrounding them thus seem to be important for holding these two water molecules in a proper position, in order to form the proper hydrogen-bond network connecting the bicarbonate to the stromal side. These residues include D1-S268, D1-Y246 for the 1st water molecule, and D1-E244, D2-T243 for the 2nd water molecule. Changes in one of these residues may therefore disturb the positions of the water molecules, and thereby disrupt the proper hydrogen-bond networks. D2-K264 is not hydrogen-bonded to any of the two water molecules, but is hydrogen-bonded to D2-E242, and also close to D1-E244, one of the residues hydrogen-bonded to the 2nd water molecule.

Alteration of D2-K264 may therefore perturb the orientation of D2-E242, resulting in an effect on the position of the 2nd water molecule.

We know that there is another short H-bond network that connects Q_B to the stromal surface, which is composed of D1-S264 and D1-H252. D1-H252; it is located in a small dent in the stromal surface and is hydrogen-bonded to a water molecule directly, which is further hydrogen-bonded to another water molecule. A number of additional water molecules are found in the vicinity of these water molecules, indicating that D1-H252 is located in a highly hydrophilic area. Thus, protons may also be easily taken from this area of the stromal surface and transferred to Q_B through D1-H252 and D1-S264. In view of the previous functional studies [142], it is plausible to suggest that the first proton to protonate Q_B^- is taken up through D1-H252 and D1-S264, and the second proton is transferred *via* bicarbonate to D1-H272 and D1-H215, and, finally to Q_B through the H-bond network (see Fig. 16, and its legend). HCO_3^- that must become CO_3^{2-}, after giving up its proton to Q_B^{2-}, may get its proton back from the stroma *via* D1-E244 [158, 159]. However, further functional studies are required to prove or disprove the order of these protonation events. In addition, since there are indications for a change of the bicarbonate coordination to the NHI from bidentate to monodentate upon electron transport from Q_A towards Q_B [150, 156], there might be alternative proton paths newly created by possible accompanying conformational changes.

In view of the above picture of the environment and plausible function of bicarbonate, we recommend comparative biochemical and biophysical studies on appropriate site-directed mutants of D1-E244; D1-Y246; D1-S268; D2-T243; D2-E242; and D2-K264.

4. Uniqueness of role of bicarbonate in oxygenic photosynthesis

The requirement of PSII for bicarbonate (carbonate) has been observed at the level of intact leaves, isolated thylakoids and PSII-enriched membrane fragments from plants, algae, and cyanobacteria, but never in the RCs of anoxygenic photosynthetic bacteria (see Sections above and references therein). It appears, therefore, that by being a ligand to the NHI between Q_A and Q_B, and binding to amino acids of the D1 and D2 proteins of PSII in these organisms, bicarbonate/carbonate plays an unique role only in oxygenic photosynthesis: it stabilizes the Q_A-NHI-Q_B structure of the PSII RC, and, thus, allows efficient electron transport and protonation of Q_B^- *via* certain amino acids around Q_B (Fig. 16). We ask: Why does the PSII RC have, unlike its bacterial cousin, a bicarbonate ion liganded to its NHI? The simple answer is that it may have a regulatory function here in PSII electron flow. Under normal conditions, bicarbonate may be bound and function in protonation events, as discussed above; however, when the plant is exposed to drought, high light and high temperature, the stomata may close, leading to a decrease of the internal [CO_2]. Similar decreases in [CO_2] are expected in algae and cyanobacteria that do not have stomata [164]. This would lead to a decrease in [HCO_3^-] limiting PSII activity.

The effect of bicarbonate depletion within PSII is not only on the electron acceptor side, but also on the donor side, although the exact location (or binding site) responsible for this effect of HCO_3^- is not known [9, 14, 20]. The effect of HCO_3^- on the water-oxidizing side of PSII has also been seen *in vitro* in all oxygenic organisms (higher plants, algae, and cyanobacteria) (for details see Section 2). There is, however, lack of observations of this effect in intact organisms. Many experimental data obtained on isolated PSII membrane fragments and PSII core preparations are consistent with a unique role of HCO_3^- in initiating and/or facilitating assembly of the inorganic core of the OEC from OEC-depleted PSII RCs (e.g., arising as a result of disassembly of the OEC under stress conditions or when newly synthesized) and Mn^{2+} ions (reviewed in [61]). There are also indications for the functioning of HCO_3^- in the assembled OEC [14]. Thus, for instance, newer data suggest that mobile (loosely bound or even non-bound) bicarbonate may facilitate deprotonation of the Mn_4CaO_5 cluster (opposite to the protonation reactions assigned for the 'acceptor-side' HCO_3^-) [32, 34]. By "picking up" the protons that are produced during water splitting, HCO_3^- *per se* or in concert with CA may play a regulatory function against over-acidification of the lumen in the proximity of the water-oxidizing site, and by this, protect the OEC against destabilization and predisposition to photoinhibition.

5. Bicarbonate and evolutionary development of the O_2-evolving Photosystem II

All O_2-producing photosynthetic organisms (cyanobacteria, green algae, and plants) have the same Mn_4CaO_5 inorganic core and very similar RC core proteins forming the basis for PSII capable of catalyzing oxidation of water. The available geological and geochemical data indicate that nature created this single type of enzyme as early as 3.2 Ga or as late as 2.4 Ga ago [165-168]. The role of bicarbonate (CO_2) in the evolutionary development of the first O_2-evolving cyanobacteria-like organisms is obvious, since the presence of HCO_3^-/CO_3^- bound between Q_A and Q_B in the RC is unique, as it exists only in oxygenic photoautotrophs, whereas it is absent in all anoxygenic photosynthesizers [122, 123]. The coupling of bicarbonate as a ligand to facilitate Q_B^- protonation and, thus, the electron transfer in the first O_2-producing organisms *via* replacement of the Glu ligand in anoxygenic bacterial RCs (as first suggested in [124]; for further details, see Section 3.1.3.1) could be simply an additional evolutionary step from anoxygenic towards oxygenic photosynthesis [169].

There are also indications for a key role of Mn-bicarbonate complexes in the evolutionary origin of the water-oxidizing inorganic core of the OEC of PSII [59, 170]. The unique capability of bicarbonate to form easily oxidizable complexes with Mn ions has been demonstrated in numerous electrochemical and EPR studies (see, for instance, [62, 64, 65]). Since the oxidation potentials of the Mn^{2+}-HCO_3^- complex (520-680 mV) were found to have close values to the midpoint redox potentials of the primary electron donor (P) in the RCs of non-oxygenic bacteria, Dismukes et al. [170] suggested, that these complexes (which could be formed under much higher concentrations of dissolved CO_2 (HCO_3^-) in the ancient ocean than at present) were probably used as a source of electrons by some Archean anoxygenic bacteria, - ancestors to the first oxygenic cyanobacteria. Results obtained both on wild type contemporary purple bacteria [171] and on mutants of *R. sphaeroides* with modified midpoint redox potentials of the P/P$^+$ RC couple [33] support this idea by showing that the formation of the Mn^{2+}-bicarbonate complexes stimulate electron donation from Mn^{2+} to type II RCs of these anoxygenic bacteria.

6. Concluding remarks

As proved by recent X-ray crystallography studies of PSII [16, 17], in the cyanobacterial RC, there is only evidence for a single bound bicarbonate at the NHI. The evidence for “bicarbonate” as ligand to the quinone-iron complex derived from a large body of data makes it clear that there is a role for this ligand *in vivo*. The presence of bicarbonate as a bidentate ligand to the NHI bridging Q_A and Q_B is now firmly established [16, 17]. This set the key stone to a huge body of studies that have established a role of bicarbonate in facilitating proton transfer and, thereby, accelerating electron transfer between Q_A, Q_B and from Q_B into the PQ-pool; the absence of bicarbonate might down-regulate this electron transfer step. Since this action prevails in all oxygenic organisms, the structural and functional role of bicarbonate has arisen very early in evolution. There is also an effect on the reoxidation of PQH_2. Comparative biochemical and biophysical studies on site-directed mutants of tyrosines near the HCO_3^- binding site is expected to provide key information on the mechanistic role of bicarbonate in these reactions.

There is another, though less well defined role of bicarbonate on the donor side of PSII. A particular binding site close to the Mn_4CaO_5 cluster is absent in the high-resolution structure [17]. Since the roles of HCO_3^- as a mobile substrate of PSII or as a direct tightly bound ligand to the Mn_4CaO_5 cluster are excluded by numerous studies, a direct involvement of HCO_3^- in the water-oxidizing process can now be ruled out. There is, however, undeniable evidence for an essential role of HCO_3^- in the process of photoactivation. Further experiments are required to evaluate the possibility of HCO_3^-

involvement in the deprotonation reactions of the OEC. The indirect effects of HCO_3^- on water oxidation (such as, protection against thermoinactivation, photoinhibition, protein extraction, and treatments with some reductants) need to be studied and characterized further. Moreover, one should clarify whether HCO_3^- ions have the same function on the donor side of PSII in intact photosynthetic systems.

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Figure legends

Fig. 1. Conversion of inorganic carbon species including acid-base ionization/dissociation constant (pK_a) values for hydrogen carbonate (bicarbonate) anion. See text for further details.

Fig. 2. CO_2 (HCO_3^-) effect on the rate of the Hill reaction as was found by Otto Warburg and Günter Krippahl [4] in isolated kohlrabi grana suspended in 0.1% KCl. The measurements were performed in the presence of 2.1 mg quinone as electron acceptor under argon (closed symbols) or argon + 1.4% CO_2 (v/v) (open symbols) in the gas phase.

Fig. 3. Schematic representation of PSII in higher plants and green algae (only core proteins are shown) and two sites (acceptor and donor) where bicarbonate (HCO_3^-; hydrogen carbonate) has effects. While *the acceptor side bicarbonate* is known to bind to the NHI (Fe^{2+}) between Q_A and Q_B, the exact location of *the donor side bicarbonate* is unknown. The acceptor side bicarbonate may also be bound to the NHI in the form of carbonate (CO_3^{2-}). In cyanobacteria the sites of HCO_3^- effects are the same, but some components of PSII are different (for further details see [15]). The pathway of the electron flow through PSII is shown by black arrows. Other abbreviations: D1 and D2, the reaction center proteins; P680, the reaction center Chl molecule; Chl_{D1}, the primary electron donor on D1; $Pheo_{D1}$, the primary electron acceptor on D1 (pheophytin); Chl_{D2} and $Pheo_{D2}$, symmetrically related cofactors on D2 (inactive branch; do not participate in linear electron transfer through PSII); Mn_4CaO_5, inorganic core of the OEC; Y_Z (on D1) and Y_D (on D2), the redox active tyrosine residues; PQ, mobile plastoquinone molecule; CP43 and CP47, Chl-protein complexes of 43 and 47 kDa; LHC-II, light-harvesting complex II; PsbO (33 kDa), PsbP (23 kDa) and PsbQ (17 kDa), extrinsic proteins of PSII; Cyt *b*559, redox active cytochrome *b*559.

Fig. 4. O_2 yield obtained on the third flash (Y_3) as a function of the dark-time between the second and the third flash (Δt_{23}) as measured by Stemler et al. [23] in dark-adapted HCO_3^--depleted chloroplast suspensions in the presence and the absence of bicarbonate. The frequency of the main flash train was 1 Hz. Y_3 values were normalized with respect to the steady-state O_2 yield. Open triangles: HCO_3^--depleted chloroplast suspensions were injected onto the Pt electrode to final Chl concentration of 0.3 mg ml^{-1}. The measurements were performed in buffered medium, which contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer (pH 6.8), 20 µg ml^{-1} of ferredoxin, and 0.5 mM $NADP^+$. Closed circles: the same as above but after re-addition of 10 mM $NaHCO_3$. Adapted and modified from [23].

Fig. 5. First experimental evidence for the action of bicarbonate on the electron acceptor side of PSII reported by Wydrzynski and Govindjee in 1975 [24]. A comparison of variable Chl *a* fluorescence on concentration of HCO_3^- and an inhibitor of PSII DCMU. (**A**) HCO_3^--depleted chloroplasts at various concentrations of $NaHCO_3$. (**B**) Non-HCO_3^--depleted chloroplasts at various concentrations of DCMU. Before the measurements, the samples were incubated in the dark for 5 min. Note that the fluorescence induction curves obtained under HCO_3^--free conditions look like the one obtained after addition of 10^{-6} M DCMU. Fluorescence was measured at 685 nm upon excitation with a broad blue light at a Chl concentration of 12.5 µg ml^{-1}. Modified and adapted from [24].

Fig. 6. Increase of DCMU-induced Chl *a* fluorescence as a function of flash number in HCO_3^--depleted (closed squares), HCO_3^--depleted plus 20 mM $NaHCO_3^-$ (open squares), and control (open circles) spinach chloroplast suspensions as measured by Govindjee et al. [104]. Other conditions: [Chl] = 20 µg

ml^{-1}; [DCMU] = 5 μM. The measurements were done in the presence of 1 mM NH_2OH used as an artificial electron donor. Modified and adapted from [104].

Fig. 7. Isolation of the photosynthetic electron transfer chain into several segments (1, 2, and 3) by using artificial electron donors and acceptors in combination with specific inhibitors of electron carriers (modified from [105]). Abbreviations: DPC, diphenylcarbazide; SM, silicomolybdate; BQ, benzoquinone; DAD, diaminodurene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea); MV, methyl viologen; Cyt *f*, cytochrome *f*; PC, plastocyanin; P700, RC Chl of PSI; A_0, and A_1, primary electron acceptors of PSI; FeS, iron sulfur centers of PSI. Other abbreviations are as in Fig. 3.

Fig. 8. Flash-induced kinetics of proton release as measured by Khanna et al. [107] in control and CO_2-depleted spinach thylakoids. (**A**) Proton release kinetics monitored by absorption changes of neutral red (NR) at 524 nm. Signals represent a difference between two transient signals (signal obtained in the absence of imidazole minus signal obtained in the presence of imidazole). The assays were performed in a medium containing 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM $K_3[Fe(CN)_6]$, 0.3 μM nonactin, 10 μM NR, and 1.3 mg ml^{-1} bovine serum albumin (BSA) at pH 7.0. (**B**) Proton release kinetics as indicated by absorption changes of bromocresol purple (BCP) at 574 nm. Reaction medium (pH 6.4) contained 20 mM KCl, 2 mM $MgCl_2$, 0.5 mM $K_3[Fe(CN)_6]$, and 20 μM BCP. In both cases the final concentration of Chl in reaction mixture was 10 μg ml^{-1}. For illumination, saturating single-turnover flashes ($t_{1/2}$ = 15 μs) were used, and the obtained signals were averaged over 10 flashes. Dark time between flashes was 10 s. Modified and adapted from [107].

Fig. 9. ^{14}C-labelled atrazine binding to CO_2-depleted (open squares), CO_2-depleted plus 20 mM $NaHCO_3$ (closed circles), and control (open circles) pea membrane thylakoids as reported by Khanna et al. [108]. The data were represented as plots of double reciprocal (mg Chl/nM bound atrazine) *vs.* 1/[free atrazine]. Thylakoids were incubated at 23ºC with various concentrations of ^{14}C-labelled atrazine. The amount of bound atrazine was calculated from the difference between the total radioactivity added to the thylakoids and the amount of free atrazine found in the supernatant after centrifugation. Modified and adapted from [108].

Fig. 10. Double reciprocal plot of the ferricyanide Hill reaction rate (v_{Hill}) as a function of the bicarbonate concentration in the absence (closed circles) and presence (open circles) of 100 nM ioxynil in pea thylakoids as reported by Vermaas et al. [109]. The samples were incubated with bicarbonate for 2 min. The measurements of O_2 evolution rates were done in the presence of 0.5 mM $K_3[Fe(CN)_6]$. Ioxynil was added 3.5 min prior to the measurements. Adapted and modified from [109].

Fig. 11. The rate of 2,6-dichlorophenolindophenol (DCPIP) reduction measured by Blubaugh and Govindjee [114] in CO_2-depleted thylakoids as a function of the equilibrium CO_2 (Panel **A**) and HCO_3^- (Panel **B**) concentrations. The reduction rate of DCPIP was calculated from the decrease in absorbance at 600 nm and normalized to the control rate. The control rates (in µmol (DCPIP$^{\mathrm{red}}$) mg (Chl)$^{-1}$ h^{-1}), estimated separately for each curve (pH value) by adding 2.5 mM HCO_3^- to the CO_2-depleted samples, were the following: 209 at pH 6.31 (open squares); 212 at pH 6.54 (open diamonds); 191 at pH 6.67 (open circles); and 192 at pH 6.87 (open triangles). $NaHCO_3$ was added 3 min prior to illumination. Inset in Panel **A**: the effect of the equilibrium $[HCO_3^-]$ on the Hill reaction, with the $[CO_2]$ held constant

at 0.1 mM. Inset in Panel **B**: the effect of the equilibrium [CO_2] on the Hill reaction, with the [HCO_3^-] held constant at 0.2 mM. Modified and adapted from [114].

Fig. 12. First detection of formate-induced release of CO_2 from spinach thylakoids as measured by MIMS by Govindjee et al. [53]. The addition of formate (to 100 mM) induced a rapid increase in the CO_2 signal (which corresponded to a formate injection artifact, *i.e.* formate blank) followed by a slow release of CO_2 from thylakoids (left). Repetitive addition of formate to the same sample induced only the initial rapid CO_2 release related to artifact of formate injection (right). CO_2 was continuously monitored at $m/z = 44$. The measurements were performed at 20°C and pH 6.5 in the presence of external carbonic anhydrase (final concentration of 0.5 µg/ml) in order to facilitate the equilibration between inorganic carbon species. Modified and adapted from [53].

Fig. 13. EPR data showing the effect of HCO_3^- removal on the acceptor side of PSII as reported by Nugent et al. [145]. (**A**) EPR spectra of the Q_A-NHI region ($g = 1.82$) in dark-adapted PSII particles from the cyanobacterium *Phormidium laminosum* upon 5 min illumination at 77 K in the absence (spectrum *1*) and the presence of 100 mM formate (spectrum *2*). (**B**) EPR spectra of the NHI (Fe^{3+}) region ($g = 6$) of the dark-adapted PSII particles from spinach in the absence (spectrum *1*) and the presence (spectrum *2*) of 100 mM formate. For further details see [145]. Modified and reproduced from [145].

Fig. 14. Probing for binding sites of HCO_3^-/CO_3^{2-} in PSII by isotope ratio MIMS in spinach PSII membrane fragments. These data confirmed the formate-induced release of CO_2 reported earlier by

Govindjee et al. (see Fig. 12) and demonstrated that all released CO_2/HCO_3^- originates from the electron acceptor and none from the donor side of PSII. (**A**) The addition of formate (to final concentration of 100 mM; black arrows) to PSII membranes at pH 6.3 and 20ºC induced a slow release of CO_2 (detected at *m/z* = 44) which was much above the artifact caused by injection of formate into the buffer with no samples (compare traces *1* and *2*). Destruction of the possible binding site (the Mn_4CaO_5 cluster) *via* the addition of strong reductant NH_2OH (to final concentration of 7.5 mM; open arrows) does not lead to a release of CO_2/HCO_3^- (compare traces *3* and *4*). During the reduction NH_2OH is known to produce N_2O. In order to shift the signal of N_2O from *m/z* = 44 to *m/z* = 46, and thus to avoid possible overlay of the CO_2 and N_2O signals the ^{15}N-labelled NH_2OH was used for these experiments. (**B**) CO_2 release upon formate addition (to 100 mM) to 'control' PSII membranes (trace *1*) is the same as in case of PSII membranes without the Mn_4CaO_5 cluster (due to pre-incubation with 80 mM NH_2NH_2 for 75 min) (trace *2*). CO_2 was detected at pH 6.3 and 20ºC as $C^{16}O^{18}O$ at *m/z* = 46 due to ^{18}O-enrichment with $H_2{}^{18}O$ (3%). (**C**) Formate-induced release of CO_2 (trace *1*) compared with the absence of CO_2 release upon injection of NH_2OH (trace *2*) as detected at *m/z* = 48 at pH 6.0 and 20ºC. To get the highest possible sensitivity the experiments were performed with high ^{18}O-enrichment level (~65%). All measurements were done in the presence of externally added carbonic anhydrase (to a final concentration of 3 µg/ml) to facilitate equilibration between CO_2 and HCO_3^- and by this to allow the detection of all dissolved inorganic carbon as CO_2 along. Modified and adapted from [41].

Fig. 15. (**A**) Structure of a PSII monomer determined at a 1.9 Å resolution [17]. View from the direction perpendicular to the membrane normal. Dashed lines represent the cytroplasmic (stromal) and lumenal surface of the membrane, respectively. Color codes: Green, D1; yellow, D2; cyan, CP47, dark pink, CP43, red, PsbL, light pink, PsbO, light blue, PsbU. The blue ball in the middle represents the NHI, and

a magenta molecule above the NHI represents bicarbonate. (**B**) An enlarged view of the NHI and bicarbonate region shown in Panel **A**. The color codes are the same as for **A**.

Fig. 16. Hydrogen-bond networks around Q_B, the NHI and bicarbonate. Blue lines represent coordination bonds, and dashed lines in cyan indicate hydrogen-bonds. Arrows in dashed, black lines indicate possible flow of protons towards the Q_B molecule. One of the protons (say the first one) may be picked up through D1-H252 and D1-S264 to protonate Q_B^-, while the second one may be trasferred throught bicarbonate, D1-H272, and D1-H215 to Q_B (see Section 3.2 for more details). Figure based on data of Umena et al. [17].

Figure 1

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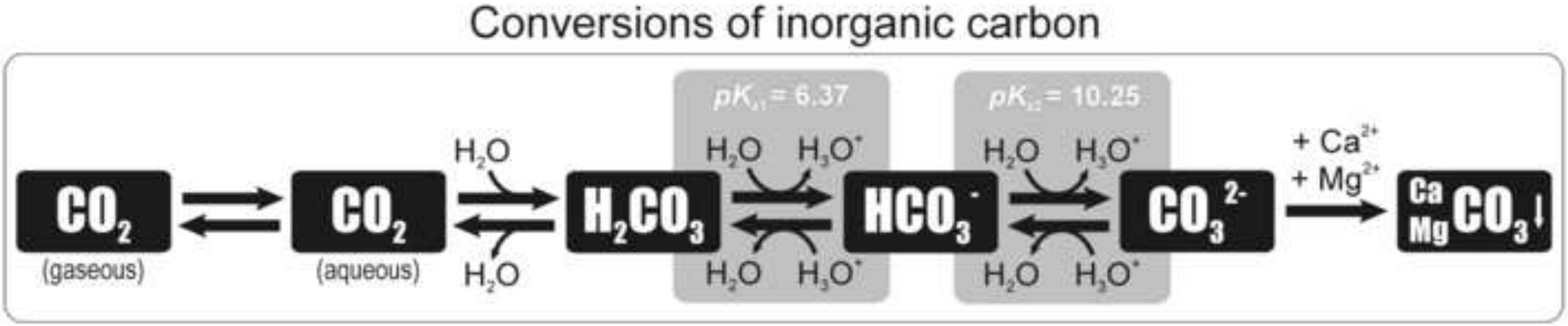

Figure 2

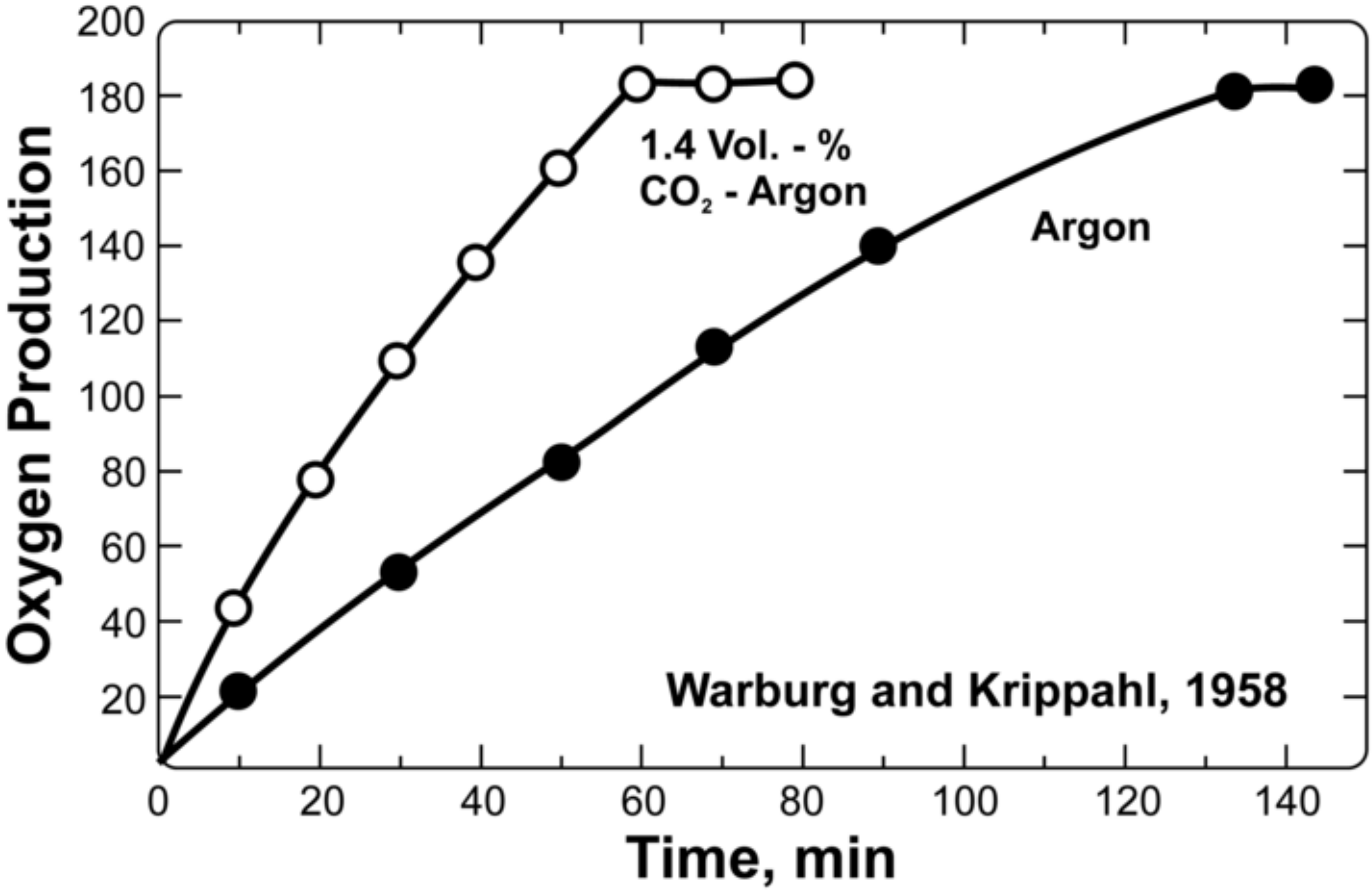
Oxygen Production
200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
Time, min
1.4 Vol. - %
CO$_2$ - Argon
Argon
Warburg and Krippahl, 1958

Figure 3

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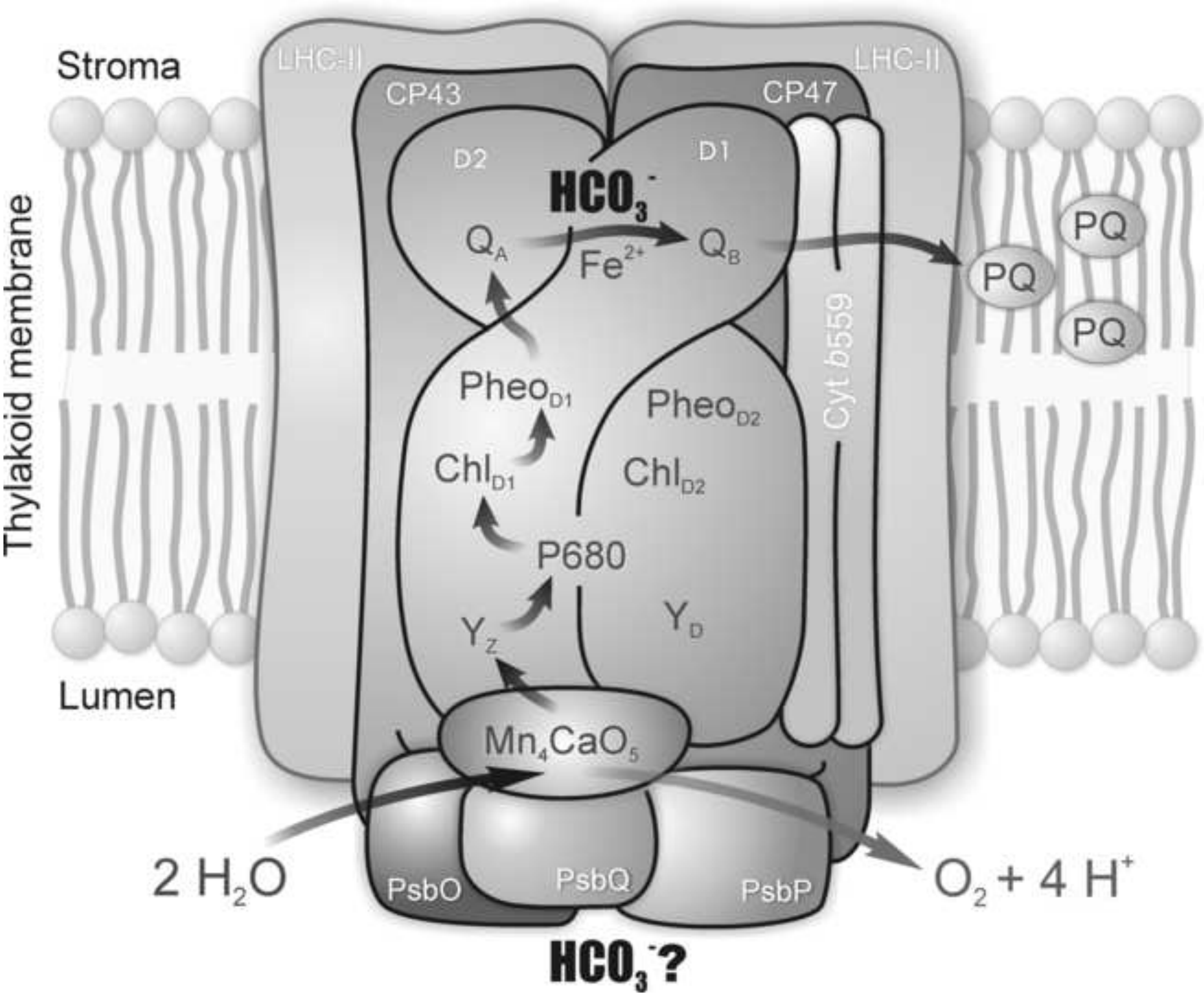

Figure 4

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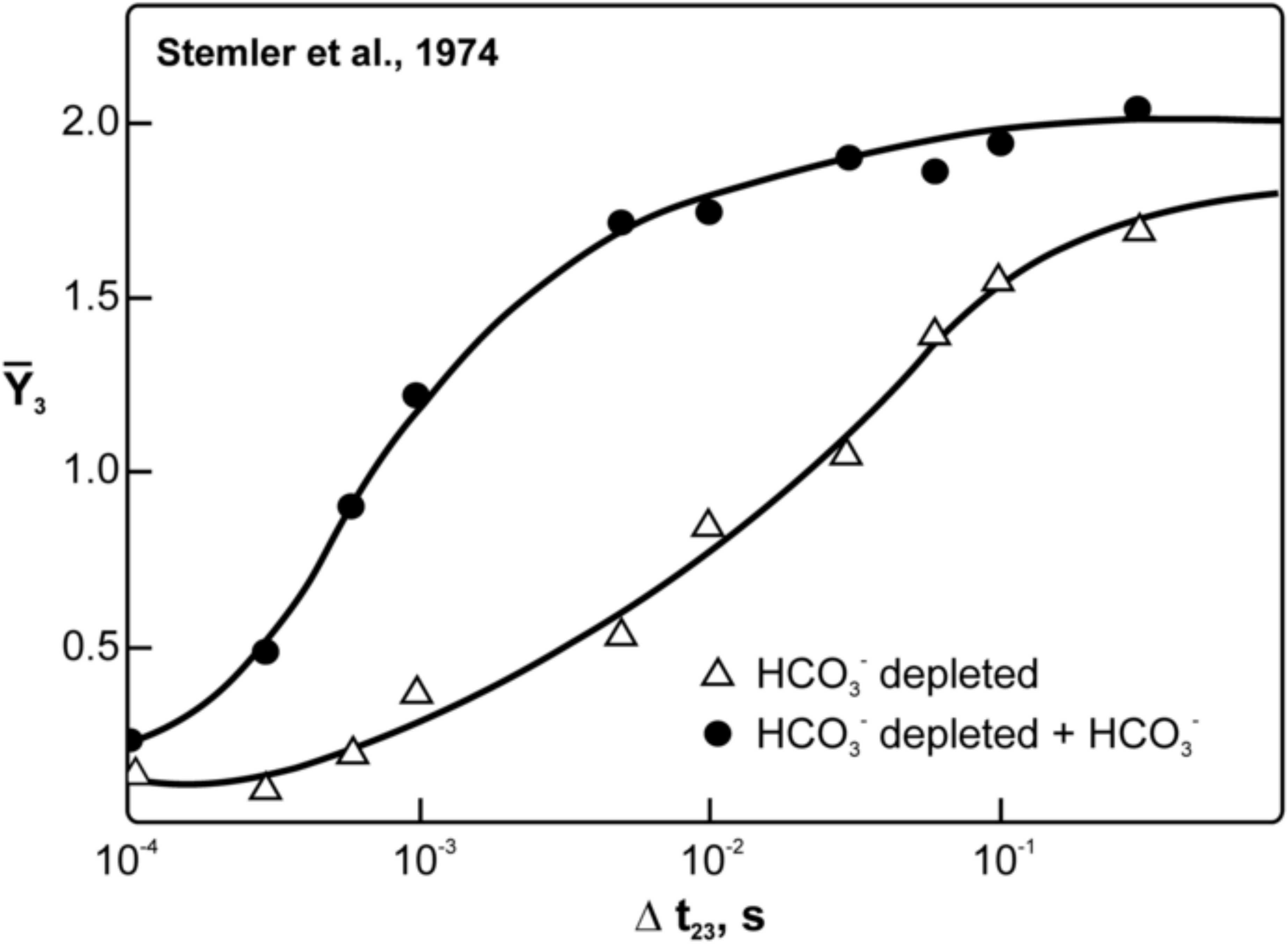

Figure 5

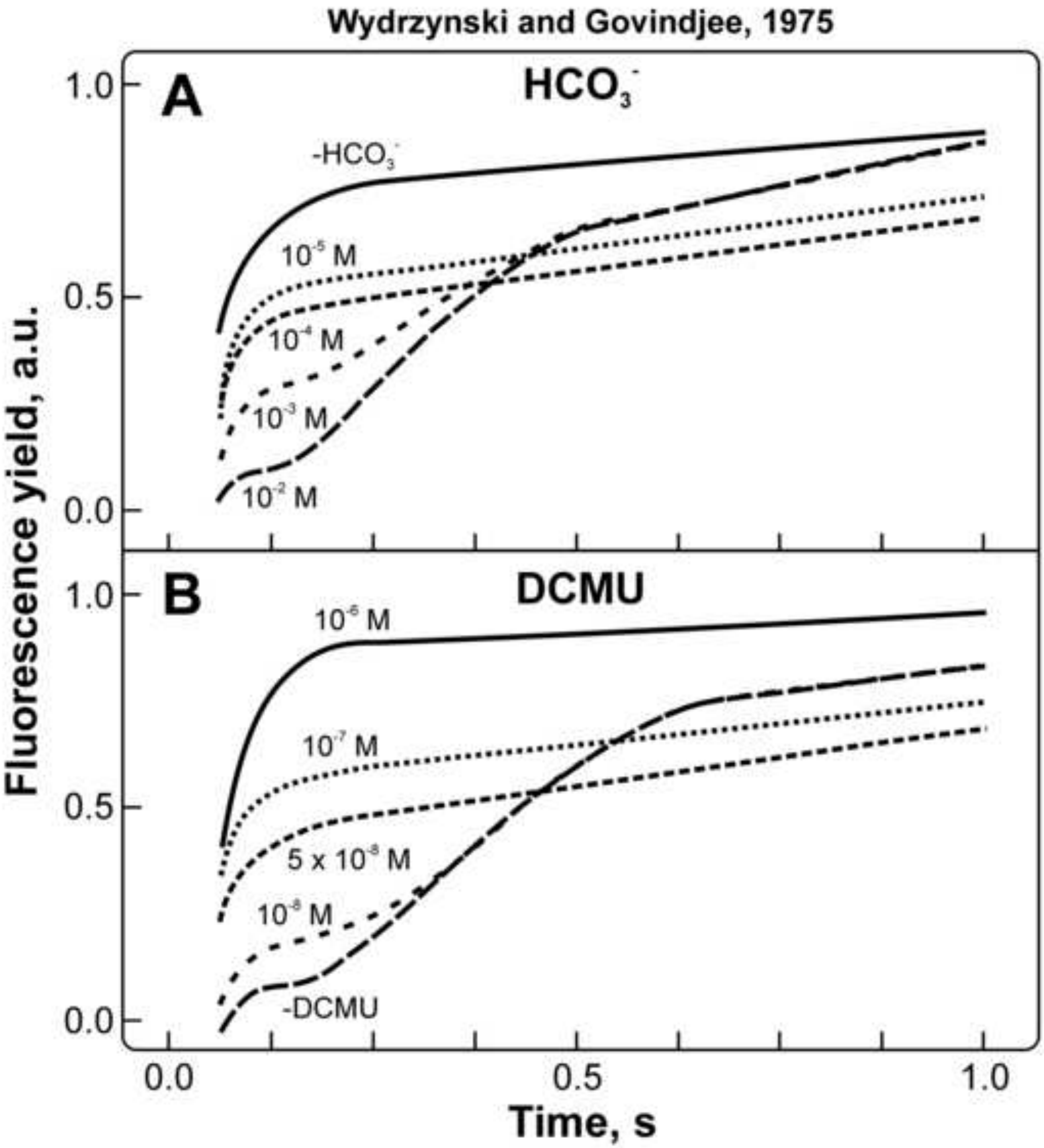
Wydrzynski and Govindjee, 1975
A
HCO_3^-
$-HCO_3^-$
10^{-5} M
10^{-4} M
10^{-3} M
10^{-2} M
B
DCMU
10^{-6} M
10^{-7} M
5 x 10^{-8} M
10^{-8} M
-DCMU
Fluorescence yield, a.u.
1.0
0.5
0.0
1.0
0.5
0.0
Time, s
0.0
0.5
1.0

Figure 6

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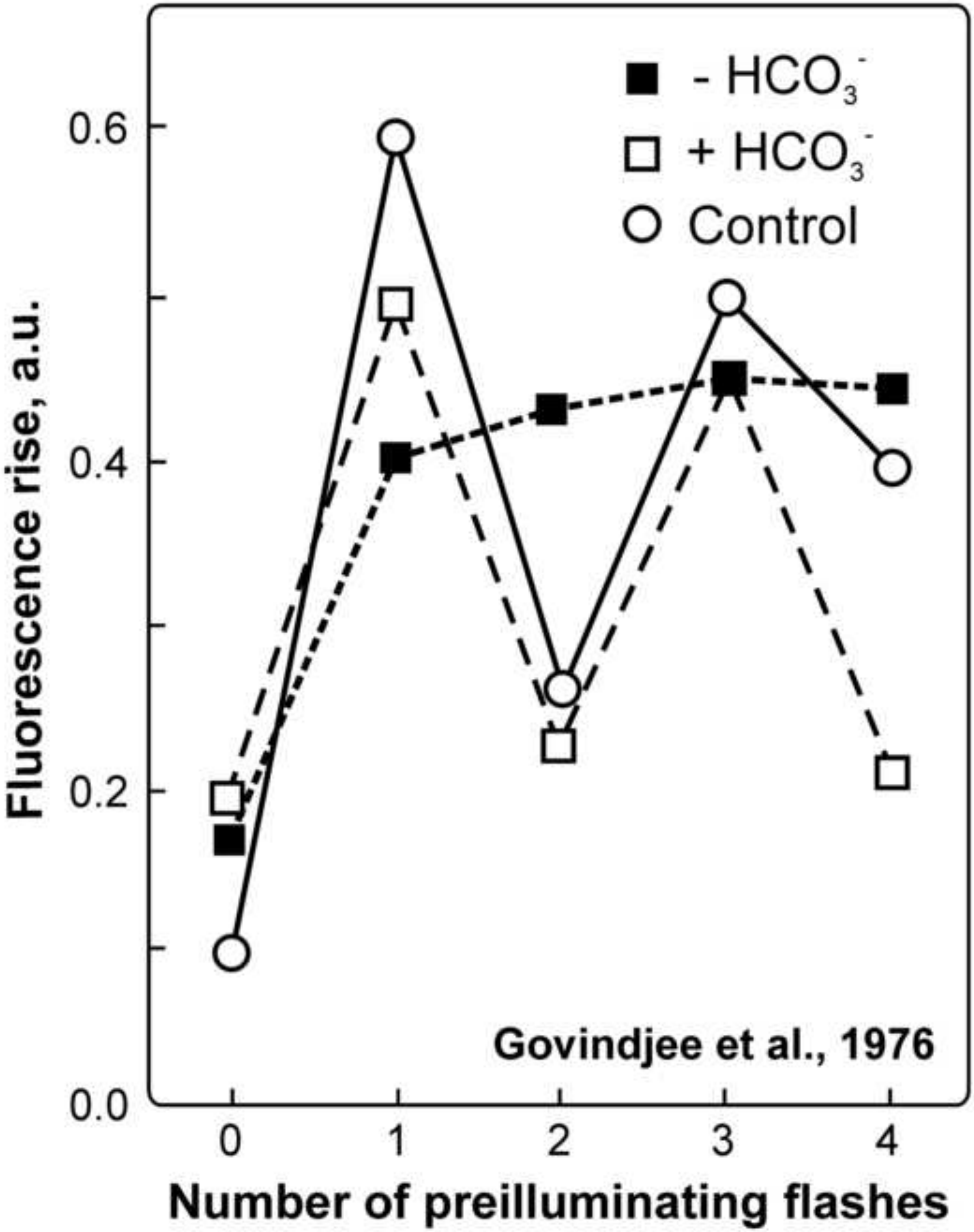
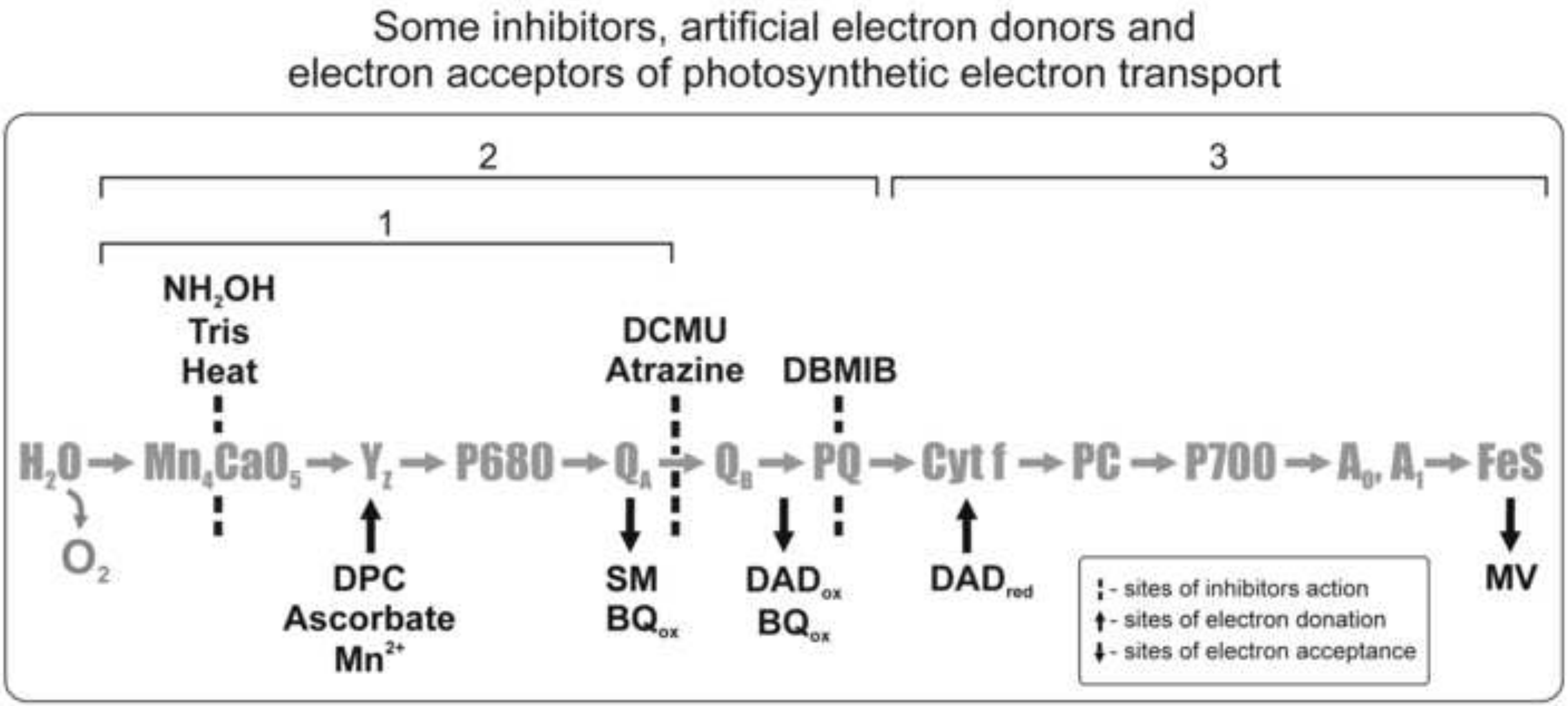
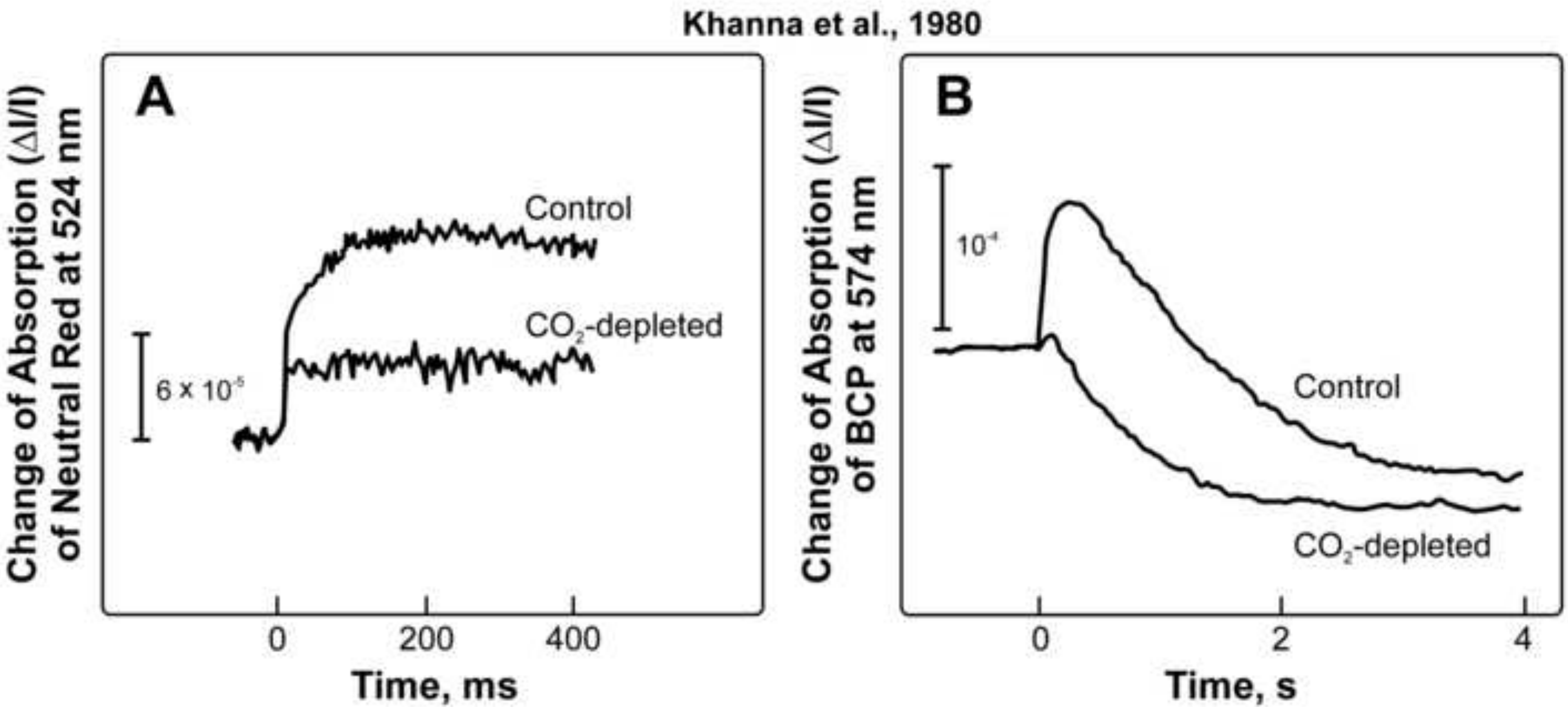


Figure 7



Some inhibitors, artificial electron donors and electron acceptors of photosynthetic electron transport

Figure 8



Khanna et al., 1980

Figure 9

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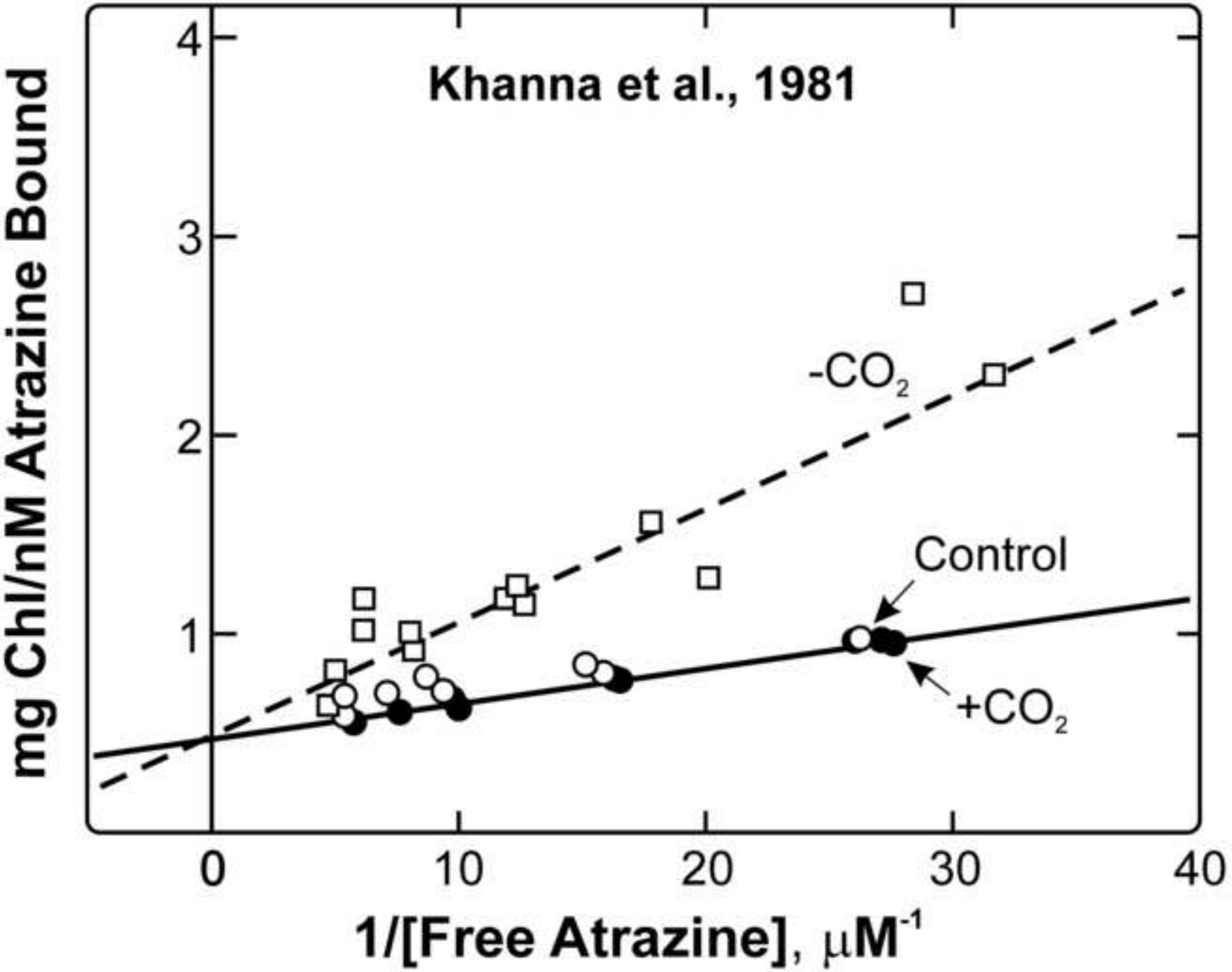

Figure 10

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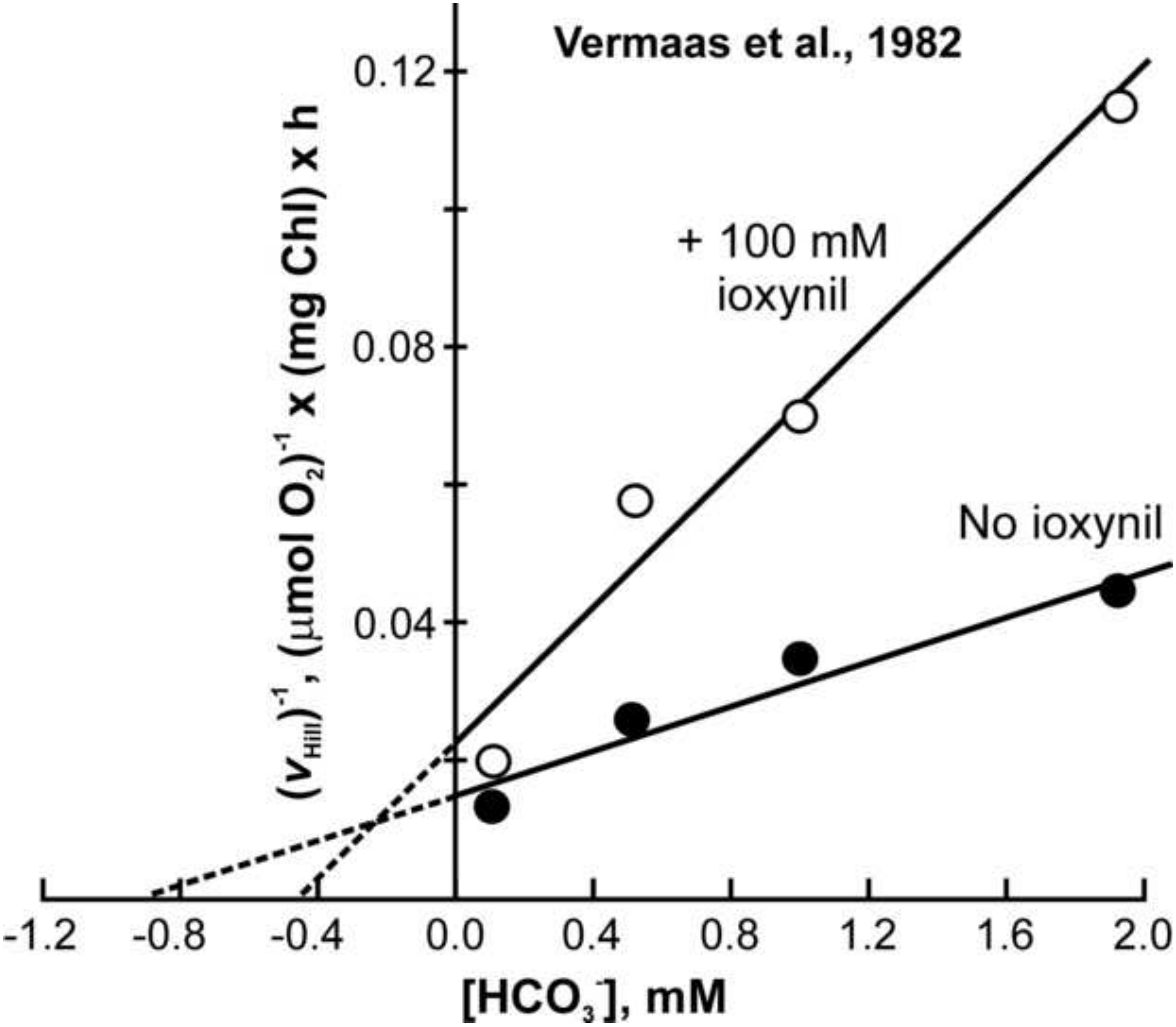

Figure 11

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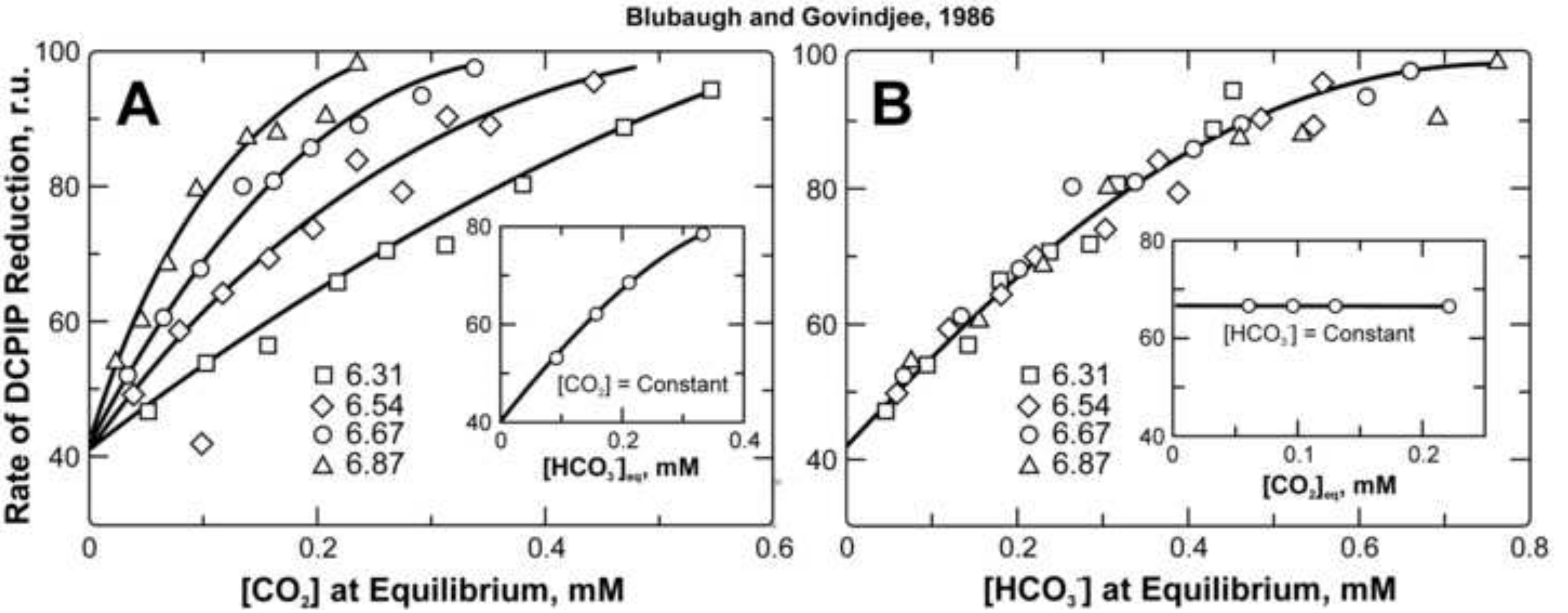

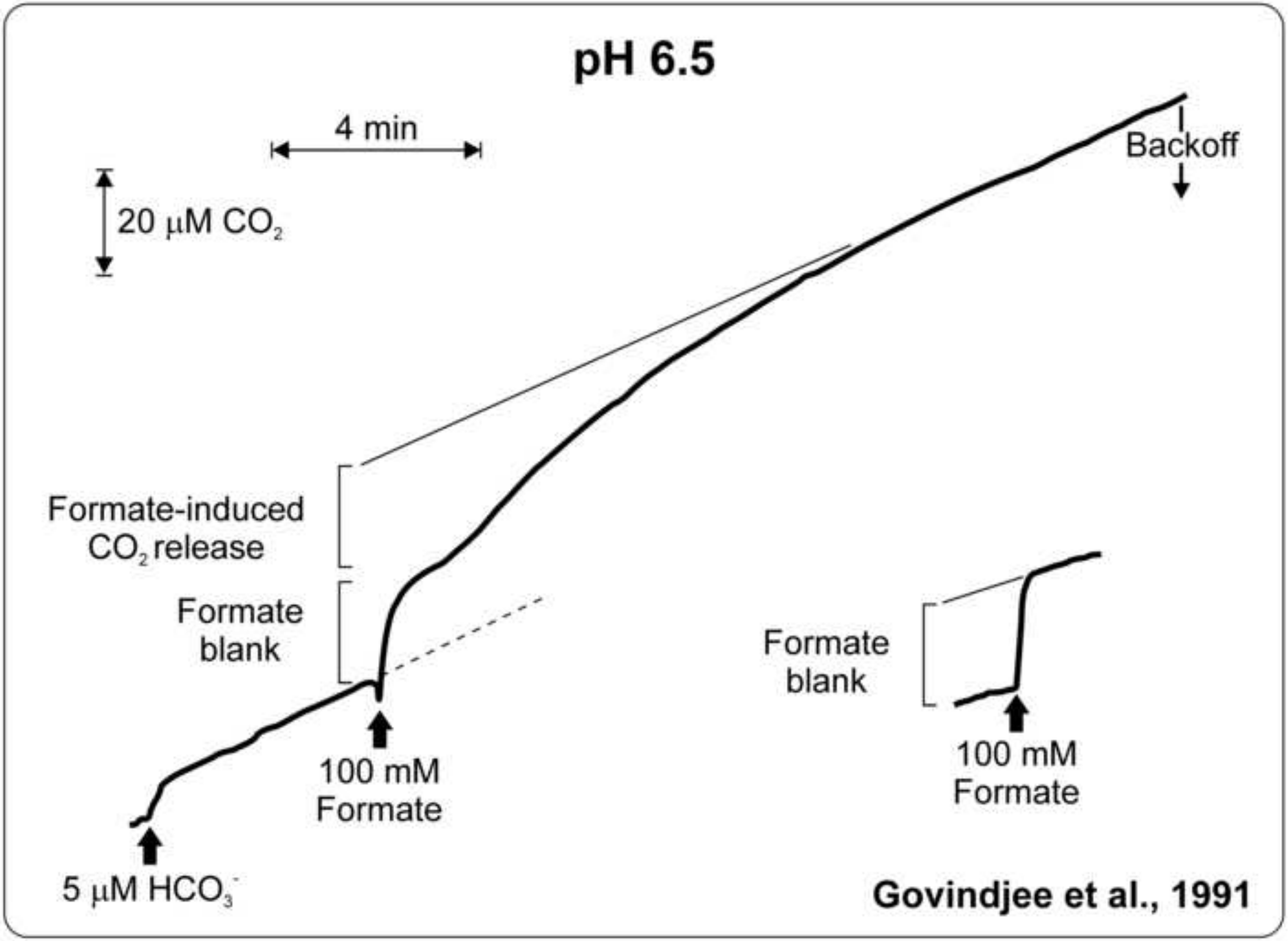
pH 6.5
4 min
20 μM CO₂
Backoff
Formate-induced CO₂ release
Formate blank
100 mM Formate
5 μM HCO₃⁻
Formate blank
100 mM Formate
Govindjee et al., 1991

Figure 13

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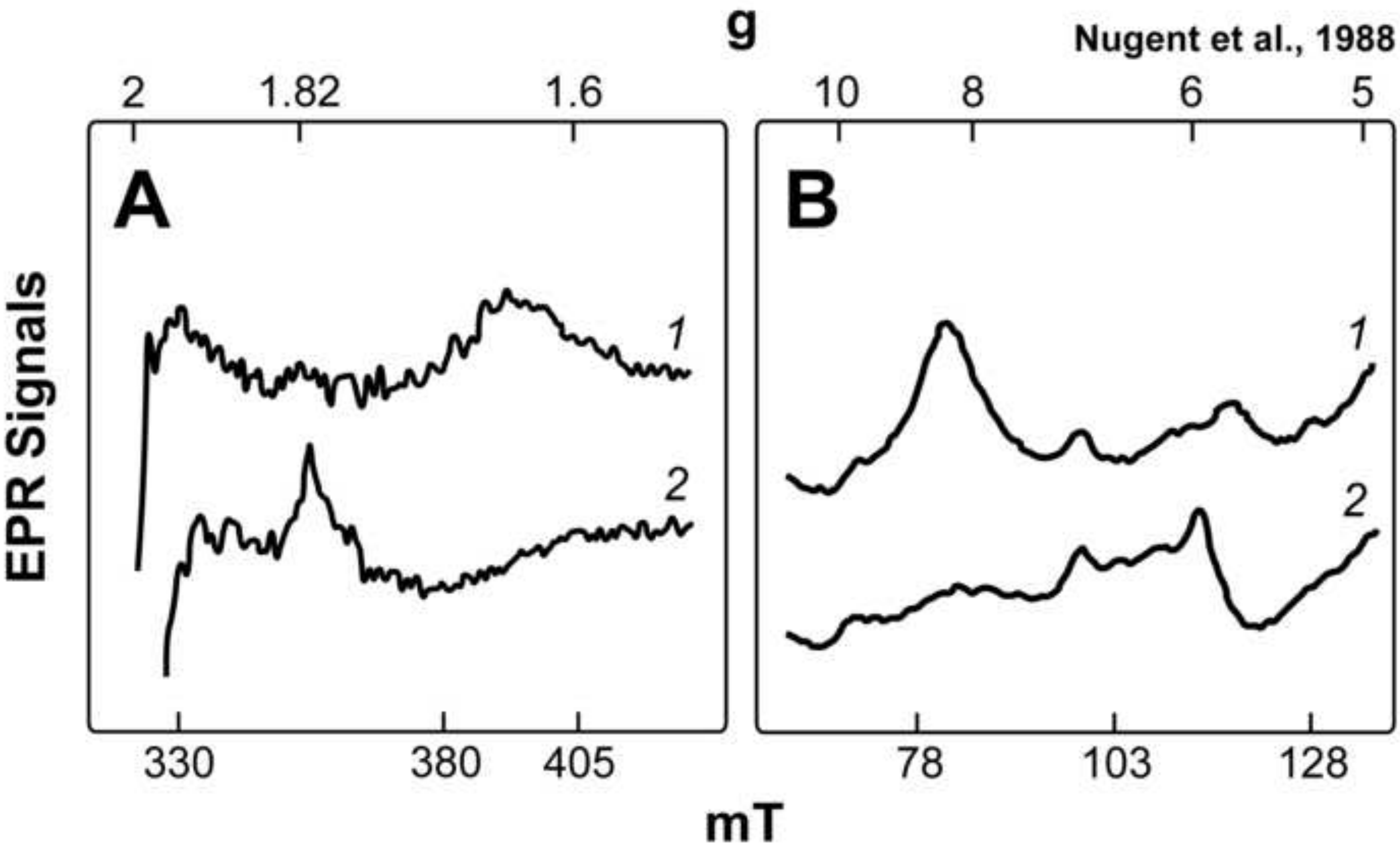

Figure 14

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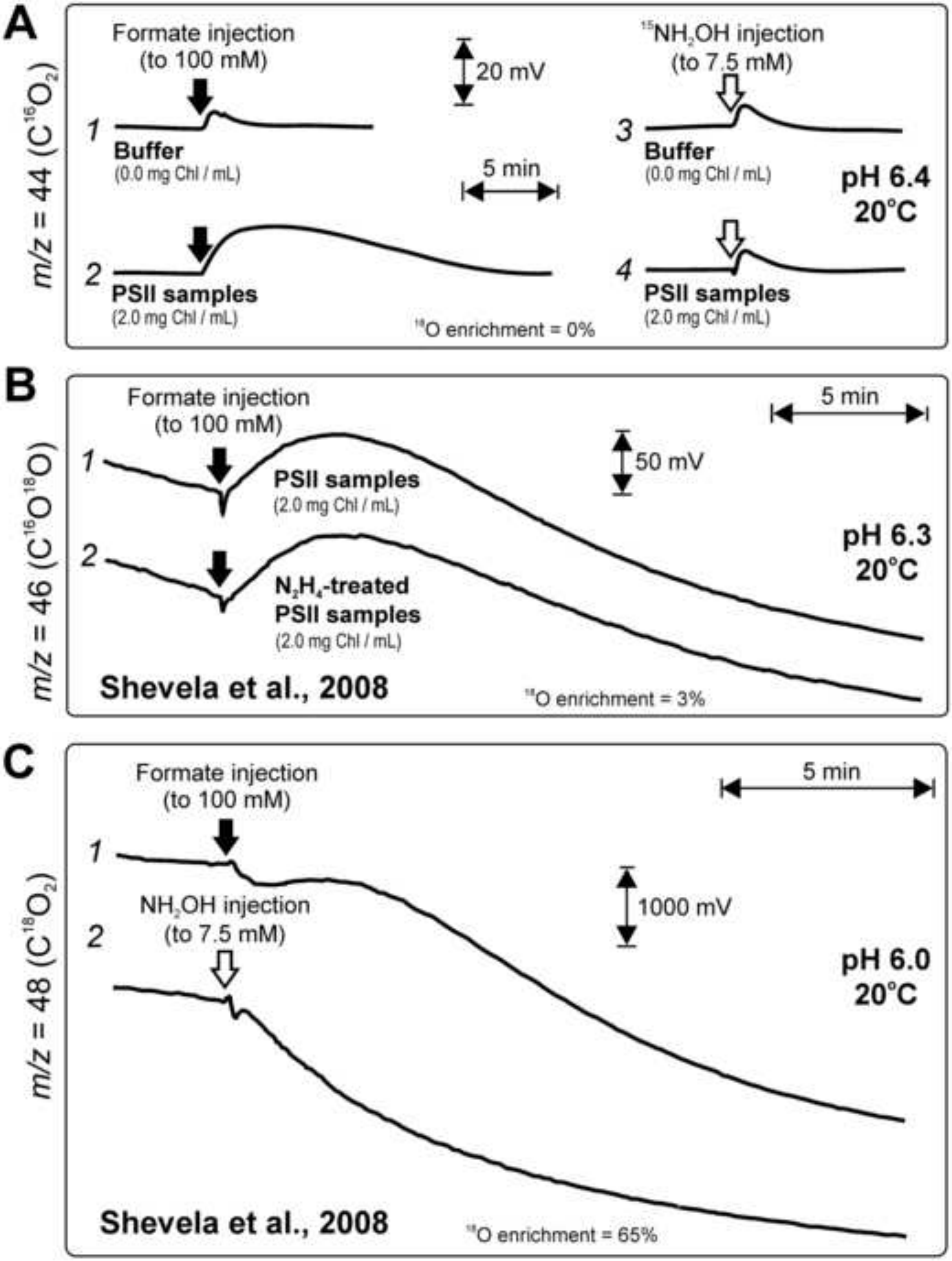

Figure 15

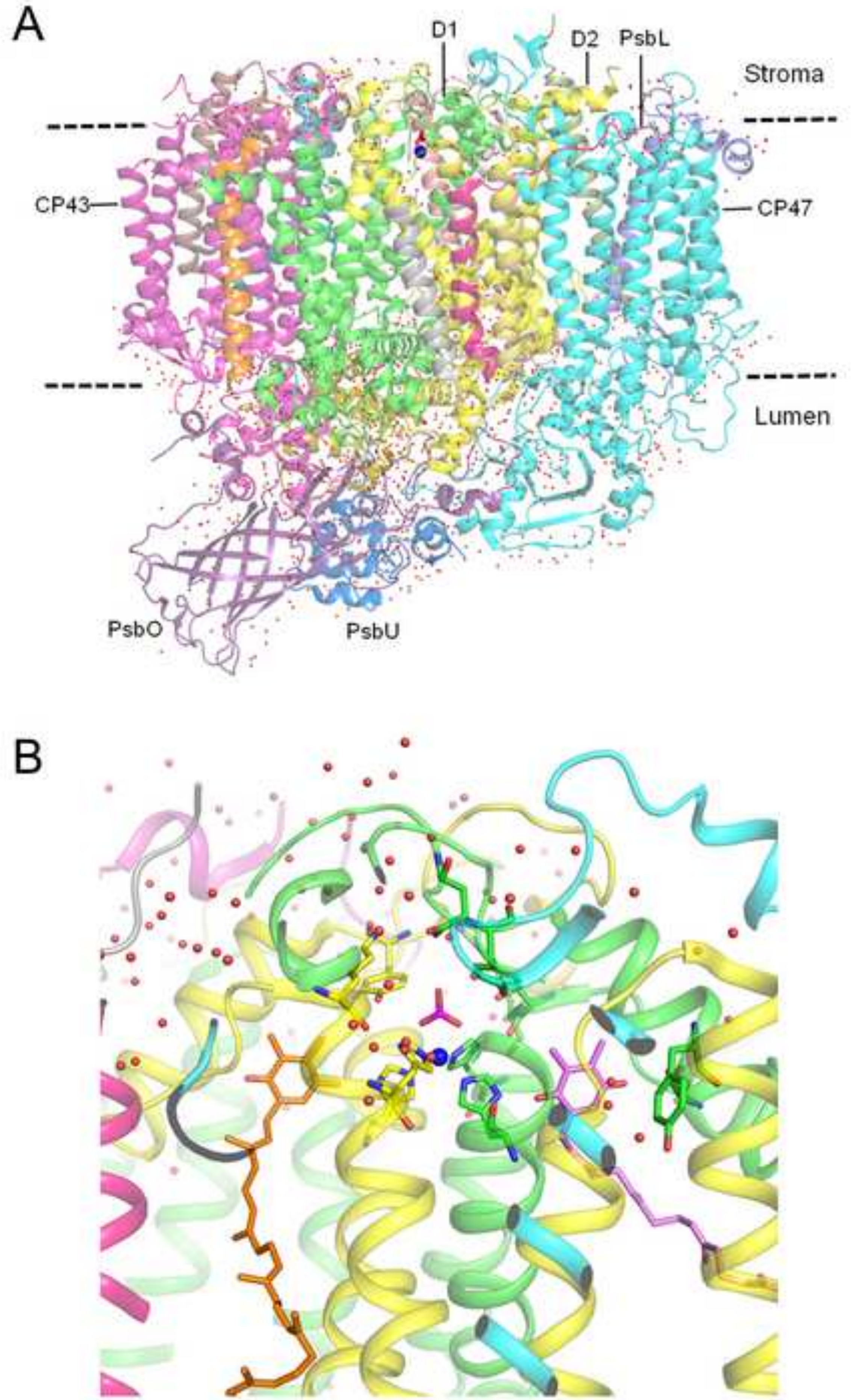

Figure 16

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