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BICARBONATE † EFFECTS ON THE ELECTRON FLOW IN ISOLATED BROKEN CHLOROPLASTS

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I. Introduction

From the beginning of the history of photosynthesis, researchers believed that carbon dioxide or bicarbonate is involved in the process of photosynthesis. The 'discoverer' of photosynthesis, Priestley [1], observed that a 'green matter', deposited on the walls of

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† Except in Section III, CO_2 and bicarbonate have been used interchangeably without implying which species is the active one.

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenol indophenol; DLE, delayed light emission; $NADP^+$, nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl) aminomethane; Chl, chlorophyll.

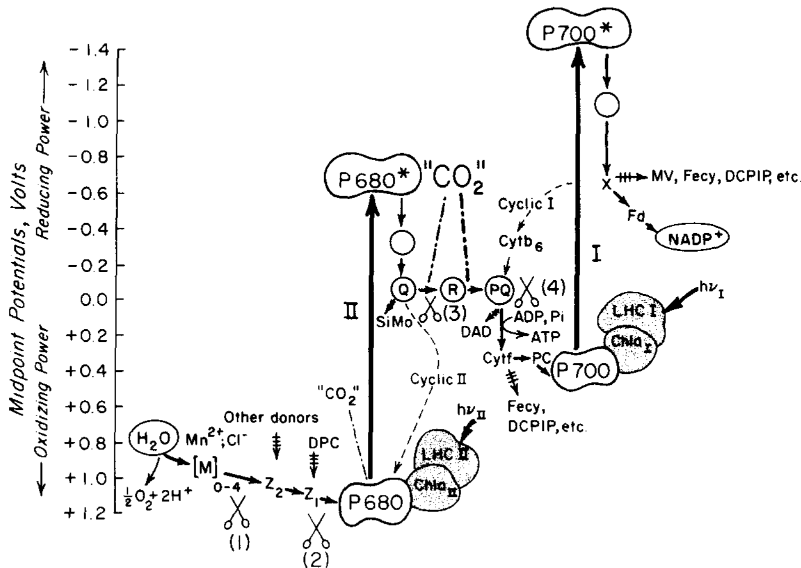


Fig. 1. Electron flow pathway from H_2O (the ultimate electron donor of photosynthesis, see lower left portion of the figure) to $NADP^+$ (see upper right portion of the figure). The two photoreactions are initiated by light ($h\nu$) absorbed in pigment System I and pigment System II. Pigment System I is suggested to consist of light-harvesting complex I (LHC I), chlorophyll a_I (not to be confused with the reaction center chlorophyll *a* in Professor Witt's terminology; here, it is a complex that belongs to the antenna system) and *P*-700 (the reaction center chlorophyll *a* which may be a chlorophyll *a* dimer of some sort). Pigment System II is suggested to consist of light-harvesting complex II (LHC II), chlorophyll a_{II}, and *P*-680 (the reaction center chlorophyll *a*, which may also be a chlorophyll *a* dimer of some sort). (There is no information as to the reasons for the difference in the red absorption peaks (at 680 or 700 nm) of the two reaction centers.) Excitation of *P*-680 and *P*-700 lead to the production of their excited singlet states (*P*-680* and *P*-700*). This is followed by the oxidation reduction reactions. Both *P*-680 and *P*-700 are oxidized (*P*-680^+ and *P*-700^+ are formed) and the electrons are delivered to the real primary electron acceptors of the two systems; in light reaction II, the first identified electron acceptor is Q (which has been shown recently to be a quinone molecule). In light reaction I, the first identified electron acceptor is labelled 'X' and it may be some sort of iron-sulfur protein. In pigment System II, *P*-680^+ is re-reduced in the dark by water through a series of intermediates labelled M_{0-4}, Z_2 and Z_1. Here M may be a manganese-containing protein having an ability to accumulate up to at least 4 positive (oxidizing) equivalents, Z_2 and Z_1 may be components containing quinone and/or tightly bound manganese. Z_2 is, in all likelihood, monitored by an electron spin resonance signal labelled II_{vf} (vf stands for very fast). It is possible that a 10 000-dalton polypeptide may be involved in Z_1. In pigment System I, *P*-700^+ is re-reduced in the dark by the reduced Q (Q^-) through a series of intermediates labelled as R (a 2-electron acceptor quinone), PQ (plastoquinone pool), cyt *f* (cytochrome *f*) and PC (a copper protein, plastocyanin). The reduced X (X^-) transfers its electron to $NADP^+$ via Fd (ferredoxin). A cyclic reaction around photosystem I (labelled cyclic I) may involve cytochrome b_6 (cyt b_6), and a cyclic reaction around photosystem II (labelled cyclic II) may involve cytochrome b_3. The electron transport inhibitors mentioned in this review are shown to act as sites labelled with scissors (X): (1) alkaline Tris-washing (or heat treatment) which blocks electron flow from H_2O to Z; (2) hydroxylamine which blocks electron flow from Z_1 to *P*-680; (3) the herbicide diuron (DCMU, see list of abbreviations) which blocks electron flow between Q and R; and (4) 2,5-dibromo-3-methyl-6 isopropyl-p-benzoquinone which blocks electron flow out of plastoquinone. Diphenylcarbazide (DPC) is suggested to donate electrons somewhere on the water side; silicomolybdate (SiMo) is suggested to accept electrons from Q^-; oxidized diaminodurene (DAD_{ox}) is suggested to accept electrons from plastoquinone; and ferricyanide (Fecy) or dichlorophenol indophenol (DCPIP) can accept electrons, depending upon experimental conditions, either from the plastoquinone-cytochrome *f* region or from the 'X' in System I. Methyl viologen (MV) accepts electrons only from 'X'. This current scheme is based on data of a large number of investigators (too numerous to cite here; see refs. 7–9).

water containers, formed bubbles of pure 'dephlogisticated air' (that is, oxygen). Ingen-Housz [2] discovered the importance of sunlight in this process, but, it was Senebier [3] who noted that the production of 'dephlogisticated air' by plants depends on the presence of 'fixed air' (carbon dioxide). In 1796 Ingen-Housz [4] translated the whole photosynthetic process from the phlogiston language into the language of the new chemistry, founded by Lavoisier:

$$CO_2 + H_2O \xrightarrow[\text{green plant}]{\text{light}} O_2 + \text{organic matter} \quad (1)$$

Today, the role of carbon dioxide in photosynthesis is obvious: without CO_2 the plant cannot perform the process of photosynthesis. Carbon dioxide is the substrate for the enzymatic reaction involved in its reduction to carbohydrate. Thus, CO_2 plays a role in the latter, dark part of the photosynthetic process not directly related to oxygen evolution [5].

In the primary reactions of photosynthesis, two light reactions in series are driven by two photosystems [6]. (See Fig. 1; for complete and detailed picture of the present day concepts on photosynthesis, see Govindjee [7], Barber [8] and Trebst and Avron [9].) Light reaction II leads to the oxidation of water, thus evolving oxygen (left bottom, Fig. 1), and the transfer of electrons from the water to an intermediate pool between Photosystem II and Photosystem I (middle Fig. 1). Photoreaction I transfer electrons from this intermediate pool to nicotinamide adenine dinucleotide phosphate ($NADP^+$) (right top, Fig. 1). The Hill reaction [10], i.e. O_2 evolution by isolated chloroplasts illuminated in the presence of an oxidant, such as ferricyanide or methylviologen, is a convenient way to measure part of this electron transport. While it was assumed for a long time that CO_2 did not play a role in the Hill reaction, Warburg and Krippahl (ref. 11, see also ref. 12) discovered in 1958 that the Hill reaction was dependent on the presence of CO_2. Although there are several effects in photosynthesis called the Warburg effect, we shall call the CO_2 effect on the Hill reaction the *Warburg effect* in this review. Warburg et al. [13] considered it as a proof of their theory that oxygen arises from the splitting of CO_2 and not H_2O. However, Warburg convinced very few. An effect of CO_2 on the Hill reaction, however, is well established now. It is this effect of CO_2 (or bicarbonate) on the Hill reaction in isolated broken chloroplasts, which will be discussed in this review. It will be shown here that bicarbonate (or CO_2) is not involved in O_2 evolution steps, as suggested by Warburg, but it is needed for the electron flow from the oxygen-evolving system to the plastoquinone pool (see ref. 14 for a review on plastoquinones; middle, Fig. 1). Whether this involves a regulatory mechanism in vivo remains to be ascertained.

II. The ability of bicarbonate to stimulate the Hill reaction and photophosphorylation in isolated chloroplasts: the Warburg and the Punnett effects

An effect of CO_2 on the Hill reaction in isolated chloroplasts was first claimed by Boyle [15] in 1948. He demonstrated oxygen production by ground-up spinach leaves, which were given *p*-benzoquinone. Since this gas production was absent when he added KOH into the center well of the Warburg vessel, he believed CO_2 was necessary for this oxygen production. These observations were confirmed by Abeles et al. [16] but were demonstrated to be artifacts. When alkali was not present in the vessel's center well, the rate of the Hill reaction was the same with or without CO_2. When alkali was present, some quinone distilled from the main compartment into the alkali-soaked filter paper in

the center well. The resulting mixture consumed oxygen at a rate comparable with the rate of oxygen production in the Hill reaction. This explained the absence of net gas production in the presence of KOH, as found by Boyle.

Such an explanation of Boyle's results was given earlier by Warburg and Krippahl [11]. However, they demonstrated that in grana from kohlrabi leaves oxygen was evolved, using quinone as an electron acceptor, with a higher rate when the argon gas in the vessel contained 1.4% CO_2 (Fig. 2); this occurred also when ferricyanide was used as an electron acceptor [12]. Warburg believed this CO_2 effect to be a catalytic one. Abeles et al. [16] confirmed the stimulation of the Hill reaction by CO_2 in kohlrabi chloroplasts and showed by mass spectrometry that this CO_2 effect was 'specific' for O_2 evolution since no metabolism of CO_2 was observed. In chloroplasts, suspended in 0.5 M sucrose and 0.05 M phosphate buffer, the increase in Hill reaction rate by CO_2 was absent; when phosphate buffer was used alone the effect was about half of that in 0.01 M KCl. These authors failed to see a significant effect of CO_2 in sugar beet chloroplasts. Therefore they concluded that this CO_2 effect was not a general one.

Stern and Vennesland [17,18] showed a CO_2 requirement for the Hill reaction, as measured by O_2 evolution, in broken chloroplasts from spinach or kohlrabi. They first removed CO_2 by a prolonged incubation of the sample in the dark in Warburg vessels with KOH in the center well. The Hill reaction with ferricyanide and catalytic amounts of trichlorophenol indophenol declined with time both in the presence and in the absence of CO_2, but was invariably greater for the sample incubated without CO_2. This was not a pH effect as buffered media had been used. After adding bicarbonate to CO_2-depleted samples a large stimulation of the Hill reaction occurred. This CO_2 effect was present in chloroplasts from various plant sources and in Hill reactions with different electron acceptors [19].

Heise and Gaffron [20] found that the absence of CO_2 decreased the rate of O_2 production which accompanies the photochemical reduction of *p*-benzoquinone in the cells of blue-green alga *Anacystis* and of the green alga *Scenedesmus*. However, light counteracted the decline in the Hill reaction rate in the absence of CO_2. These authors suggested that this CO_2 effect is not an 'important' one since many different metabolic reactions have been shown to possess such dependency on traces of carbon dioxide.

Izawa [21] remarked that conditions to demonstrate a CO_2 effect on the Hill reaction varied widely. While Abeles et al. [16] used no pretreatment, but eliminated buffer from the reaction media, others used very long preincubation of the chloroplasts in the light

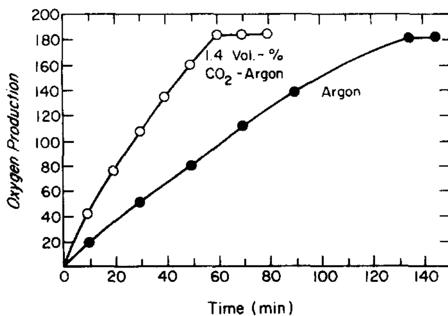


Fig. 2. Experiments with grana suspended in 0.1% KCl (3 ml vessel). Electron acceptor, 2.1 mg quinone; gas phase, Argon or Argon + 1.4% CO_2 by volume. (After ref. 11.) Note: This experiment shows that CO_2 enhances the Hill reaction.

[11] or in the dark [17,18] with CO_2-removing systems. Izawa considered these procedures rather inadequate, since they inevitably induced a considerable loss of Hill activity of chloroplasts or a pH change. Hence he added carbonic anhydrase to the reaction medium as well as KOH in the center well of the Warburg vessel. By speeding up the equilibration between free CO_2 and carbonic acid in this way, he obtained a CO_2 effect already after 15 min. The effect was larger in broken chloroplasts than in the whole ones. Also Good [22] was concerned about the very different techniques used in producing the CO_2 effect and studied conditions favorable to promote this effect. He [22,23] found that pea chloroplasts incubated for two or three hours under a CO_2-free atmosphere in a buffered medium may lose the greater part of their Hill reaction capacity. The CO_2 dependence of the Hill reaction was largely influenced by additions of anions or mixtures of anions to the incubation medium. Chloride and fluoride and, at much lower concentrations, formate and acetate, increased the dependence of the chloroplasts on CO_2. Furthermore, the rate of the CO_2-depleted system was more depressed when both chloride and acetate were used in the medium than when chloride alone was present (Fig. 3).

The finding that CO_2 dependence is correlated with the presence of small anions led Good to suggest that bicarbonate is the important species, not CO_2. Since Good did not succeed in finding an anion able to fulfill the function of bicarbonate in CO_2-depleted chloroplasts, the effect appears to be specific for bicarbonate. Phosphate, pyrophosphate, arsenate, citrate, maleate, malonate, trimethylacetate, *p*-hydroxylbenzoate, glycine and tricine did not affect the Hill reaction rate, either in the presence or absence of CO_2. Glycolate and malate were the only two ions found to have any action in relieving the

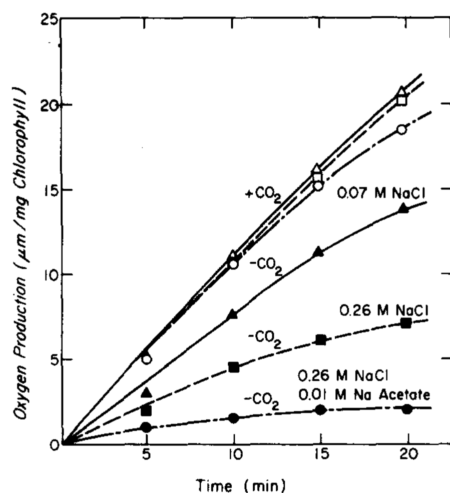


Fig. 3. The influence of chloride and acetate on the CO_2 dependence of the Hill reaction. Pea chloroplasts (126 μg chlorophyll) were incubated for 2.5 h at 16°C in 0.05 M phosphate buffer containing the indicated concentrations of sodium chloride and sodium acetate plus $1.35 \cdot 10^{-7}$ mol dichlorophenol indophenol. Final pH, 6.6; final volume, 1.9 ml. The center well of the manometer vessels contained either KOH or CO_2 buffer. The concentration of CO_2 in the gas phase in equilibrium with this CO_2 buffer (65 parts 2.0 M $KHCO_3$: 35 parts 2.0 M K_2CO_3) was approximately 1.1%. 20 μmol of potassium ferricyanide in 0.1 ml water were tipped into the vessel from the side arm after the incubation period and before the period of illumination. The 3 lower curves illustrate the progress of ferricyanide reduction in the absence of CO_2. The 3 upper curves are their controls with CO_2. (After ref. 22.) Note: This set of experiments shows that the presence of both NaCl and sodium acetate allows a better depletion of CO_2.

inhibition caused by CO_2 removal, but their effect was very small. In CO_2-depleted chloroplasts uncoupler did not stimulate the rate of the Hill reaction with ferricyanide, as they did in the presence of CO_2. This was also found by Stern and Vennesland [18]. Hence, Good concluded that the CO_2 effect was located in the electron transport pathway and not in the phosphorylation mechanism.

West and Hill [24] showed that the reduction of dichlorophenol indophenol, as well as that of ferricyanide, decreased in CO_2-depleted pea chloroplasts. The stimulatory effect of CO_2 was greater in broken chloroplasts than in whole ones confirming the results of Izawa [21]. In agreement with the results of Good [22] and Stern and Vennesland [18], West and Hill found the uncoupler NH_4Cl to have no effect in CO_2-depleted chloroplasts; however, a slight stimulation was observed after the readdition of bicarbonate. The CO_2 requirement for the Hill reaction is found to be maximal when electron transport is uncoupled [25].

The study of the CO_2 effect was extended by Punnett and Iyer [26] and Punnett [27]. In isolated oat chloroplasts, without pretreatment, suspended in buffer, addition of bicarbonate caused enhancement of ferricyanide reduction both in the presence and in the absence of ADP. They discovered that photophosphorylation was enhanced at pH 7.0 *; moreover, the ATP : $2e^-$ ratio increased upon the addition of bicarbonate. This effect will be called the *Punnett effect* in this review. This enhancement of photophosphorylation by CO_2 was much greater with chloroplasts from winter-grown oats than summer-grown oats. The cyclic phosphorylation (cyclic I, Fig. 1) with pyocyanine as a cofactor responded to bicarbonate as much or more than the phosphorylation accompanying the non-cyclic Hill reaction (H_2O to ferricyanide, etc.). This led these authors to the conclusion that bicarbonate had an effect on the phosphorylation mechanism, i.e., bicarbonate increases the efficiency of formation of a 'high energy intermediate' (or ATP) resulting from electron transport.

The results of Punnett and Iyer were confirmed by Batra and Jagendorf [25]. Cohen and Jagendorf [28] observed, just as Punnett had done for oats, that the stimulation of the rate of pyocyanine-catalyzed photophosphorylation by bicarbonate is much greater in chloroplasts from winter-grown than from summer-grown spinach. They suggested this phenomenon to be related (indirectly, we assume) to the prior life history (e.g., hormonal status) of the plants. Bicarbonate stimulated ATP synthesis if added in the final ATP-forming state of either a post-illumination or an acid-base experiment. Bicarbonate also stimulated the membrane-bound Mg^{2+}-dependent ATPase of chloroplasts. Nelson et al. [29] showed that bicarbonate stimulated the residual Mg^{2+}-ATPase activity of detached and purified 'coupling factor' protein 3- to 4-fold and suggested that bicarbonate may increase the Mg^{2+}-ATPase activity of the purified enzyme by changing the conformation of the enzyme, which would result in more efficient catalytic action. W.S. Cohen (personal communication, and ref. 30) monitored the energy-dependent conformational changes of chloroplast coupling factor protein by measuring tritiation of the protein when it is energized. He found that when bicarbonate stimulates ATP synthesis, it enhances the energy-linked conformational change of the coupling factor protein.

Batra and Jagendorf [25] argued that there are two different effects of bicarbonate: the CO_2 requirement for the Hill reaction, discovered by Warburg and Krippahl [11,12],

* A fundamental point, according to Jagendorf (personal communication), about the bicarbonate stimulation of photophosphorylation is that it is only seen at a pH below the optimum, i.e. at pH 7, never at pH 8; basically, it extends the pH optimum for photophosphorylation, making it broader.

and the stimulation of photophosphorylation by added bicarbonate, discovered by Punnett and Iyer [26]. The differences between these two effects can be outlined as follows.

(1) The effect discovered by Warburg (the Warburg effect) is a requirement since the rate of the Hill reaction is decreased upon removal of CO_2 and restored by adding back bicarbonate. The Punnett effect is a stimulation, not a requirement, since depletion of the medium is not necessary. Moreover, removal of CO_2 does not inhibit photophosphorylation and cyclic electron transport, although these are stimulated by adding high levels of bicarbonate [25].

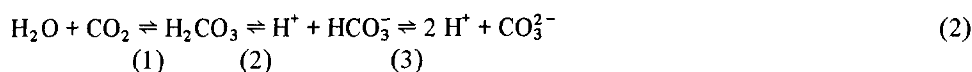
(2) The Punnett effect needs higher concentrations of bicarbonate than the Warburg effect.

(3) The Punnett effect applies to phosphorylation by pyocyanine-dependent electron flow, is independent of oxygen evolution and probably functions at some step in the phosphorylation pathway. The Warburg effect is absent in cyclic photophosphorylation [25], and probably pertains to electron transport pathways leading to oxygen evolution.

Most of the research reviewed up to this point was aimed to see if there was an effect of CO_2 on the Hill reaction or not. The magnitudes of the effects and the conditions producing them showed considerable variations. Stemler and Govindjee [31] presented in 1973 their first study on the effect of bicarbonate on the Hill reaction; they had worked out a procedure to show a large and reproducible effect of bicarbonate (by flushing chloroplasts with nitrogen in a medium containing high anion concentration at low pH), and proceeded to study the site and mode of action of bicarbonate in the electron transport chain (see Section V).

III. The nature of the active species remains unknown

In order to understand the mode of action of CO_2 on the Hill reaction, it is important to know whether the charged bicarbonate anion or the dissolved CO_2 gas is the active species promoting the effect. In water, dissolved CO_2 has the following equilibria:



$$H_2O + CO_2 \underset{(1)}{\rightleftharpoons} H_2CO_3 \underset{(2)}{\rightleftharpoons} H^+ + HCO_3^- \underset{(3)}{\rightleftharpoons} 2\,H^+ + CO_3^{2-} \quad (2)$$

In step (2), the pK of Eqn. 2 is 6.37, while the concentration of H_2CO_3 is essentially dependent upon dissolved CO_2. The pK of step (3) is about 10.8. Steps (2) and (3) are fast compared to step (1). The hydration of CO_2 or the dehydration of carbonic acid is, thus, the rate-limiting step; at pH < 8 and 18°C, a dissolved CO_2 molecule lives, on the average, about 1 min before it is hydrated (see pp. 173–179 in ref. 32 for further details).

Most experiments on the effects of CO_2 on the Hill reaction are performed at a pH above 6.37. This means that in these experiments bicarbonate is the prevailing species. Good [22] showed stimulation of the Hill reaction upon addition of bicarbonate at pH values of 6.55 and higher. Since he found that CO_2 dependence was correlated with the presence of small anions during the depletion period, he concluded that bicarbonate is the important species, not CO_2. He suggested the phenomenon to resemble ion antagonism.

Stemler and Govindjee [31] studied the effect of bicarbonate on the dichlorophenol indophenol Hill reaction in CO_2-depleted maize chloroplasts at pH 5.8 and 6.8 (Table I). At pH 5.8, the concentration of CO_2 is about 4-times the concentration of bicarbonate, while at pH 6.8 the concentration of bicarbonate is much higher than that of CO_2. Table I

TABLE I

INITIAL RATE OF DCPIP REDUCTION AS A FUNCTION OF CO_2/HCO_3^- CONCENTRATION AT pH 5.8 AND 6.8

(After ref. 31.) The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer, 39 μM dichlorophenol indophenol, and 15 μg of chlorophyll/ml of chloroplast suspension. Saturating red light was $2 \cdot 10^6$ ergs · cm^{-2} · s^{-1}. The data are the average of 5 experiments. Note: Data presented in the table characterize the CO_2 effect as a function of $[HCO_3^-]$ at two different pH values; the effect is much larger at pH 6.8 than at 5.8 (see data for 0.1 to 5.0 mM $NaHCO_3$). See Section III for discussion.

pH	$NaHCO_3$ (mM)	Rate (μmol DCPIP reduced/mg Chl · h)	$+HCO_3^-/-HCO_3^-$ (ratio)
5.8	0	17.3 ± 3.6	–
	0.1	17.6 ± 4.8	1
	0.5	23.3 ± 3.4	1.3
	1.0	24.4 ± 3.0	1.4
	5.0	38.2 ± 3.9	2.2
	20.0	78.8 ± 9.3	4.5
6.8	0	15.2 ± 2.6	–
	0.1	23.3 ± 5.1	1.5
	0.5	43.8 ± 6.5	2.4
	1.0	47.0 ± 4.9	3.1
	5.0	65.7 ± 2.7	4.3
	20.0	71.6 ± 5.1	4.7

shows that at 5 mM $NaHCO_3$, for example, the stimulation at pH 5.8 is only 2.2-fold, while at pH 6.8 it is 4.3-fold. This could suggest that bicarbonate is the active species. Khanna et al. [33], studying the effect of a suboptimal concentration of bicarbonate as a function of pH (5.8–8.0) in spinach chloroplasts, found the largest stimulation of the ferricyanide Hill reaction in the pH range of 6–7. In this range bicarbonate is the predominating species.

However, while the available data are consistent with bicarbonate being the active species, Stemler and Govindjee [31] already remarked that the evidence is not conclusive. If the affinity of the active site for CO_2 were lowered at lower pH, CO_2 should have less stimulatory effect, even though CO_2 occurs in higher concentration at pH 5.8. Moreover, while in the dark the stroma pH is between 6.8 and 8.8 [34], the stroma pH increases upon illumination and the thylakoid space attains a lower pH, well below 6.37. This means that in the thylakoid space the equilibrium is shifted towards CO_2.

In conclusion, it is still an unanswered question whether CO_2 or bicarbonate is the active species. In the process of CO_2 reduction in the Calvin-Benson-Bassham cycle, this question has been solved in favor of CO_2 as it was possible to work with the soluble enzyme ribulose biphosphate carboxylase [35]. In the case of stimulation of the Hill reaction by bicarbonate one still has to work with membrane systems. At this moment it seems rather hopeless to answer this question since the precise component to which bicarbonate binds has not yet been isolated.

IV. Is bicarbonate a source of evolved oxygen?

An early theory about the primary photochemical process in photosynthesis was that of the decomposition of carbon dioxide. Willstätter and Stoll [36] suggested that photo-

synthesis consists of two photochemical hydrogen-hydroxyl exchanges in a chlorophyll-carbonic acid complex, alternating with catalytic dismutations of the two peroxides formed by these exchanges: performic acid and performaldehyde. This theory was modified by Franck and Herzfeld [37] in order to account for energetic problems. Based on Gaffron's [38] evidence for the formation of amine peroxides in the chlorophyll-sensitized photooxidation of amines, Gaffron and Wohl [39,39a] suggested that amine peroxides may occur as precursors of oxygen in photosynthesis. (For a review of the early literature see pp. 286–293 of ref 32.)

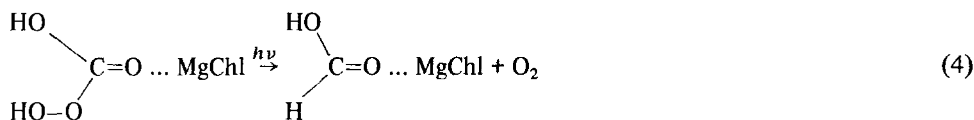
The alternate hypothesis of photochemical decomposition of water was discussed as early as 1864 [40]. However, it took a very long time before this idea became generally accepted. Although there is almost complete absence of knowledge of how water is decomposed, it is the generally accepted hypothesis today [41,42]. There are three different arguments to favor water decomposition over that of carbon dioxide: the biochemical comparison between plant and bacterial photosynthesis [43], the 'independence' of the Hill reaction from carbon dioxide [44], and the enrichment of ^{18}O in the evolved O_2 when ^{18}O-labelled H_2O is used rather than when ^{18}O carbon dioxide is used [45].

Biochemical comparison of higher plant and bacterial photosynthesis led Van Niel [43] to the following general characterization of photosynthesis:

$$CO_2 + 2\ H_2A \xrightarrow{nh\nu} (CH_2O) + H_2O + 2A \tag{3}$$

Here, H_2A is a hydrogen donor and $nh\nu$ stands for n photons or quanta. According to this equation photosynthetic bacteria gain their necessary hydrogen atoms from various reducing compounds, such as H_2S. In this case A = sulfur. In photoreduction with hydrogen in hydrogen-adapted algae, A = nothing. Consequently, in photosynthesis of higher plants evolving oxygen, A = oxygen. However, Metzner [46] dismisses Van Niel's argument since green plants, in contrast to photosynthetic bacteria, require two photoreactions to transfer electrons from the level of the primary donor (H_2O-CO_2 system) to that of the redox couple which ultimately brings about the reduction to carbohydrate. Photosynthetic bacteria require electron sources which are relatively easily oxidized; they cannot use water and are unable to evolve oxygen. The bacterial photosystem is therefore more similar to Photosystem I of green plants than the oxygen generating Photosystem II. (However, unlike Photosystem I, bacteriochlorophyll fluorescence yield changes with changes in photochemistry [47] just as chlorophyll *a* fluorescence yield changes with changes in photochemistry of pigment System II [48].)

The belief in the 'independence' of the Hill reaction from carbon dioxide stems from experiments by Hill and Scarisbrick [44] who showed that in dry leaf powders, incapable of CO_2 reduction, oxygen could be produced in the light upon the addition of an electron acceptor such as ferrioxalate; this was generally taken as the first experimental evidence to support the water decomposition hypothesis. However, there is definitely an effect of bicarbonate on the Hill reaction, as described in section II of this review. This effect led Warburg et al. [13] to resurrect the idea of a chlorophyll (Mg-Chl)-carbonic acid complex, first proposed by Willstätter and Stoll [36]. Warburg termed the complex 'photolyte' and considered it the direct precursor to oxygen upon excitation of chlorophyll by a single quantum ($h\nu$):



$$(HO)(HO{-}O)C{=}O \ldots MgChl \xrightarrow{h\nu} (HO)(H)C{=}O \ldots MgChl + O_2 \tag{4}$$

Warburg et al. [13] suggested that the CO_2 requirement for the Hill reaction reflects a catalytic function for CO_2. Specifically, they proposed that a peroxide of carbonate is the precursor of the oxygen evolved. Thus, the elimination of O_2 would yield a carbon moiety at the oxidation-reduction level of formate as the first reducing agent formed by the light. The isolated chloroplasts should not be able to retain the reduced CO_2, as in normal photosynthesis, however, and all of the reduced CO_2 is reoxidized by the Hill reagent, which also serves as the oxidant to convert CO_2 to its peroxide. Brown and Franck [49] could not find any ^{14}C fixation in illuminated chloroplasts. Vennesland et al. [50] showed that HCN inhibits the Hill reaction in a manner that suggested a reversible competition with CO_2. Since Mn^{2+} is involved in the oxygen evolving mechanism [51,52] and is also known as a cofactor for a variety of carboxylation enzymes, they suggested Mn^{2+} and CO_2 may be required in combination.

While Warburg and Krippahl [12] claimed illumination of the chloroplasts for at least 1 h to be necessary to exhaust the presence of CO_2 and precursors, Good [22] was not able to confirm this; he had illuminated chloroplasts in the precence of dichlorophenol indophenol. Good also criticized the two pieces of evidence cited by Warburg for his theory of carbon dioxide decomposition: the indispensability of CO_2 as a specific reactant in the Hill reaction and the finding of Warburg and Krippahl [12] that the Hill reaction and photosynthesis require similar concentrations of CO_2, thereby implying a common role for CO_2. Good favoured the possibility that bicarbonate is not a metabolite but could be necessary for the maintenance of structural integrity of the chloroplast system.

The requirement for CO_2 in the Hill reaction is well established now (cf. Section II of this review). If the evolved oxygen should originate from CO_2, the effect of bicarbonate on the Hill reaction must be located on the oxidizing (or water) side of Photosystem II. Only a very small effect of bicarbonate on this side has been found by Kelly and Izawa [53], and, moreover, this effect is not specific because chloride has a large effect here. As will be discussed in Section V of this review, there is now overwhelming evidence that the major bicarbonate effect is located on the reducing (or the plastoquinone) side of Photosystem II. In conclusion, although a small effect of bicarbonate on the oxidizing side of Photosystem II cannot be excluded, its major effect is located on the reducing side. This makes CO_2 a less favourable substrate for oxygen evolution than H_2O.

The third argument for the decomposition of water is the experiment with ^{18}O labelled water and carbon dioxide. Ruben et al. [45] showed that the proportion of ^{18}O in the oxygen produced by a suspension of *Chlorella* cells is equal to its proportion in water and independent of its concentration in carbonate. For a survey of early isotope studies see ref. 54, pp. 1915–1918. The concept of water decomposition was challenged by Warburg [55] and Metzner [56]. In an elaborate review of isotope experiments, Metzner [46] criticizes the results of Ruben and co-workers on grounds of rapid isotope exchange in H_2O/CO_2 mixtures, brought about by low pH and the presence of the enzyme carbonic anhydrase. Both factors could not be controlled in early experiments, using intact algal cells and long adaptation and/or collection times.

Metzner [57] measured the ^{18}O content of photosynthetic oxygen after the introduction of labelled bicarbonate to suspensions of unicellular green (*Chlorella*) and blue-green algae (see Table II). The data were integrated between the 15th and the 60th second after the addition of $NaHC^{18}O_3$. If the photosynthetic oxygen should be the product of a light-induced decomposition of water, its ^{18}O content should not exceed the ^{18}O content of the water, which was calculated not to exceed 0.2053%. The experimental data shown in

TABLE II

^{18}O CONTENT (IN %) OF THE PHOTOSYNTHETIC OXYGEN RELEASED BY ILLUMINATED CELLS

(After ref. 57.) Note: The ^{18}O content of algae, after application of $NaHC^{18}O_3$, was 0.227 ± 0.004% in contrast to the ^{18}O content of water calculated not to exceed 0.2053%. The observed higher content was used to question if the photosynthetic oxygen is a product of light-induced decomposition of water. See Section IV for discussion.

Organism	% ^{18}O after application of	
	$NaHC^{16}O_3$	$NaHC^{18}O_3$
Chlorella pyrenoidosa	0.202	0.227
Anabena variabilis	0.199	0.224
Anabena flos aquae	0.202	0.234
Microcystis aeruginosa	0.197	0.231
Average value (weighted):	0.199 + 0.001	0.227 + 0.004

Table II are, however, slightly higher. For different data and interpretations from experiments with intact cells, see Stachewski [58].

On the other hand, Stemler and Radmer [59] showed that bicarbonate is not the substrate for photosynthetic oxygen evolution 3–9 min after CO_2 addition. In their experiments, illustrated in Fig. 4, the authors used isolated broken chloroplast fragments, thus minimizing the problems of low intracellular pH and removing nearly all carbonic

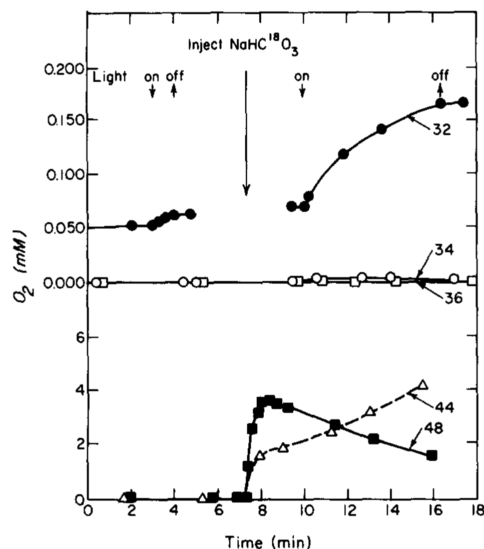


Fig. 4. ^{18}O content of dissolved carbon dioxide and photosynthetically evolved oxygen with time. The reaction mixture contained 0.175 M NaCl, 0.1 M sodium formate, 0.05 M *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer, pH 7.5, 2 mM $K_3Fe(CN)_6$, and chlorophyll, 100 μg/ml. Light from a projection lamp was filtered through 12 inches (30.5 cm) of water and a combination of C.S. 3-66 and 4-72 (3-110; λ_{max} = 580 nm) Corning filters and focused onto the reaction vessel. The intensity was about one-half of saturation. Mass numbers represented by each curve are indicated: 32($^{16}O_2$), 34($^{16,18}O_2$), 36($^{18}O_2$), 44($C^{16}O_2$), and 48($C^{18}O_2$). (After ref. 59.) Note: This experiment suggests that the source of O_2 evolution is H_2O, not CO_2.

anhydrase. As can be seen in the bottom curves of Fig. 4, the rate of $^{18}O_2$ exchange between $C^{18}O_2$ (mass 48) and H_2O, resulting in $C^{16}O_2$ (mass 44) was slow, indeed indicating the absence of carbonic anhydrase. In these experiments, the chloroplasts were depleted of bicarbonate, thus making oxygen evolution dependent on an exogenous source of this ion. As shown in the top of Fig. 4, oxygen evolution in these bicarbonate-depleted chloroplasts was low. When labelled bicarbonate was re-supplied, oxygen evolution commenced at a high rate, confirming the bicarbonate effect on the Hill reaction. At 5 minutes after CO_2 addition, $^{18}O_2$ label was still approx. 50% in CO_2; but, almost all O_2 evolved was $^{16}O_2$. Thus, the oxygen evolved after the addition of $HC^{18}O_3^-$ reflected the isotopic composition of the unlabelled water rather than the labelled bicarbonate, demonstrating that the oxygen was derived from water. (This, however, does not rule out a catalytic effect of CO_2.)

A full discussion of all the problems in experiments with ^{18}O labelled water and carbon dioxide is beyond the scope of this review. (See, however, comments by Stemler [60].) In conclusion, most evidence points to water as the ultimate source of photosynthetically produced oxygen. However, as the mechanism of oxygen evolution is still unresolved, it is wise to keep an open mind with respect to a catalytic role of an anion in oxygen evolution; this ion is most probably chloride, but bicarbonate may replace it to some degree.

V. Characterization of the bicarbonate requirement in the Hill reaction

Stemler and Govindjee [31] investigated the effect of bicarbonate to see if it is involved in the oxygen evolution mechanism. They used the following procedure to deplete chloroplasts of bicarbonate. Broken chloroplasts were isolated from various plant species by differential centrifugation and suspended in a solution containing 100 mM NaCl, 100 mM sodium formate, and 50 mM sodium phosphate, pH 5.0. The low pH and the use of high salt concentrations are crucial to the CO_2-depletion procedure. The chloroplast suspension was flushed with a stream of nitrogen gas over the sample (see ref. 61) for 10 min in the dark at room temperature. After centrifugation, the bicarbonate-depleted chloroplasts were suspended in CO_2-free reaction mixture and the rate of the Hill reaction with ferricyanide or dichlorophenol indophenol was measured. After adding bicarbonate the stimulation of the Hill reaction was recorded. This procedure yielded reproducible bicarbonate effects. Usually a 4- to 10-fold increase in the rate of the Hill reaction was observed upon addition of bicarbonate.

By studying various photosynthetic reactions in bicarbonate-depleted and bicarbonate restored chlorplasts this effect was characterized. Although at first there was an impression that bicarbonate acted on the oxidizing (water) side of Photosystem II [62], it was later established that its effect is localized on the reducing (plastoquinone) side of Photosystem II (see Sections VB and VC).

VA. Light intensity dependence: two effects

By studying the inhibitory effect of bicarbonate depletion at various light intensities, information may be obtained on its mechanism. If the inhibition is observed only at high light intensities, it means that a dark, probably enzymatic reaction is affected. If the inhibition occurs only at low light intensities, it implies that photochemical events are influenced. However, if the effect occurs at all the intensities, it may imply that at least two effects are involved, one in the photochemical and one in the dark events.

Izawa [21] reported that CO_2-stimulation of the Hill reaction in CO_2-depleted

chloroplasts was about 10 times larger at 30 000 lux than at 3000 lux. Good [22] also demonstrated a lower stimulatory effect of bicarbonate on oxygen evolution at low light intensities. Both authors, measuring oxygen evolution (the Hill reaction) manometrically, concluded that the site of the bicarbonate effect is located in the non-photochemical part of the Hill reaction.

Measuring the Hill reaction spectrophotometrically, West and Hill [24] confirmed that the ferricyanide Hill reaction is independent of removal and addition of bicarbonate at low light intensities. In contrast, they found that in dichlorophenol indophenol reduction, lack of CO_2 limits the rate up to the lowest light intensity, at which the reaction could be measured. Stemler and Govindjee [63], who measured ferricyanide reduction amperometrically, showed that the bicarbonate effect was present at all the intensities used: even at the lowest intensity where the Hill reaction could be measured, bicarbonate stimulated the rate to the same degree as at saturating intensity. However, measuring oxygen evolution, during ferricyanide Hill reaction with a Clark-type oxygen electrode, Stemler et al. [61] found the stimulation caused by resupplying bicarbonate to depleted chloroplasts to depend on light intensity. While at light saturation the stimulation was about 5-fold, it declined with decreasing intensity; at the lowest intensity used, it was less than doubled.

There is no contradiction in the data at high light intensities; there is definitely an effect on one of the dark reactions. However, the conflicting results at low light intensities are difficult to explain. While the absence of a bicarbonate effect at low light intensities points to an action site completely removed from the photochemical reactions, the presence of such an effect points to an influence on the photochemical reactions. Both possibilities were combined when it was found [61,64] that the absence of bicarbonate causes a reversible partial inactivation of Photosystem II reaction center activity, and an inhibition of electron flow from Q^- (Q being the primary electron acceptor of Photosystem II) to the intersystem electron transport pool. The conflicting results at low light intensity could be due to different reaction conditions, e.g. different degrees of CO_2 depletion of the chloroplasts (also see ref. 65). (Since the CO_2 effect at low light intensities is much smaller than at high light intensities, an inadequate depletion procedure may not show the effect at low intensities.)

VB. Electron transport: bicarbonate requirement for the electron flow from the primary acceptor of the System II to the plastoquinone pool

Experiments in which ferricyanide or dichlorophenol indophenol is used as an electron acceptor in the Hill reaction do not give much information on the site of action of an inhibition since these acceptors can accept electrons from both System I and System II depending on the experimental conditions (refs. 66–68; ref. 69 see pp. 313–315). More information can be obtained by using various combinations of electron donors and acceptors, and inhibitors, acting at various sites along the electron transport chain (see Fig. 1). For a review on electron donors and acceptors in photosynthetic electron transport see Hauska [70], and on inhibitors, see Izawa [71].

The first experiment in which an artificial electron donor was used to locate the site of bicarbonate action was reported by Stemler and Govindjee [31]. They used heat-treated chloroplasts, which were unable to evolve oxygen [72] (see scissors No. (1) in Fig. 1). Diphenyl carbazide, an artificial electron donor to Photosystem II (ref. 73; see lower left portion of Fig. 1), was added to these chloroplasts and the rate of dichlorophenol indophenol reduction in the light was measured with and without bicarbonate in bicarbonate-

depleted chloroplasts. While in normal chloroplasts the bicarbonate stimulation was 4.4-fold, it was only 1.1-fold in heat treated chloroplasts with diphenyl carbazide as a donor. (However, in the latter case the rate of dichlorophenol indophenol reduction was almost as high as in the bicarbonate restored experiment; but, this was not stressed in this paper.) At that time, the above experiments were taken to mean that the bicarbonate action is located before the site of donation of electrons by diphenyl carbazide, i.e., on the oxygen evolving side of Photosystem II. However, this conclusion had to be modified as it later became known that the use of diphenyl carbazide has problems. Harnischfeger [74] suggested that diphenyl carbazide increases the efficiency of Photosystem II in addition to its function as an electron donor. Wydrzynski and Govindjee [75] also found and drew attention to the fact that in chlorplasts treated with high concentration of alkaline Tris (see scissors No. (1), Fig. 1), to remove all oxygen-evolving capacity [76], diphenyl carbazide itself caused an enhancement in dichlorophenol indophenol reduction in CO_2-depleted chloroplasts. Furthermore, under conditions yielding a 10-fold stimulation by bicarbonate, a 2- to 4-fold stimulation by diphenyl carbazide itself was also observed for diphenyl carbazide to dichlorophenol indophenol electron flow. Thus, the absence of bicarbonate effect on this reaction, observed earlier by Stemler and Govindjee [31], could no longer be taken to mean that bicarbonate is required for the oxygen evolution step.

Kelly and Izawa [53] studied electron transport in chloride-depleted chloroplasts. These chloroplasts showed virtually no DCMU-insensitive silicomolybdate reduction. (In the presence of DCMU, silicomolybdate accepts electrons from the primary electron acceptor of Photosystem II (Q) [77,78]; for the site of DCMU action, see scissors No. (3), Fig. 1.) The reduction of silicomolybdate, as measured by O_2 evolution, was readily restored upon the addition of 10 mM chloride. Since chloride-depleted chloroplasts showed a high activity in the presence of Photosystem II electron donors, it was suggested that the chloride effect is specific for the water-splitting mechanism, labelled $[M]_{0-4}$ in Fig. 1, confirming an earlier report [79]. Bicarbonate addition to chloride-depleted chloroplasts had only a very small stimulating effect (10%) on silicomolybdate reduction, suggesting that bicarbonate can only replace chloride with a very low efficiency. Moreover, Khanna et al. [33] found no effect of bicarbonate depletion on the Hill reaction from water to silicomolybdate (Table III). Electron transport from water to oxidized diaminodurene in the presence of DBMIB was largely depressed after bicarbonate depletion. (See scissors No. (4) for the site of DBMIB action). Because oxidized diaminodurene accepts electrons at the plastoquinone pool [80] and DBMIB is an inhibitor of electron flow from the plastoquinone pool to cytochrome *f* [81], these results mean that the bicarbonate effect is located between Q and the plastoquinone pool (see middle of Fig. 1). In addition, the electron transport from reduced diaminodurene to methyl viologen in the presence of DCMU was not impaired by bicarbonate depletion, indicating that Photosystem I dependent electron transport from the diaminodurene donating site (plastoquinone?) to methylviologen is not influenced by bicarbonate; Khanna et al. [33] have further confirmed that this bicarbonate effect is not related to the effect of bicarbonate addition on phosphorylation [26], because they found that uncouplers of photophosphorylation (like NH_4Cl, gramicidine and methylamine) do not eliminate the bicarbonate effect. In fact, chloroplasts used by Khanna et al. were uncoupled to begin with. This is in agreement with the earlier results [18,22,24] and conclusions (see Section II) of Batra and Jagendorf [25] that there are two separate effects of bicarbonate.

In a completely different system, i.e., non-bicarbonate-depleted chloroplasts, Crane

TABLE III

EFFECT OF BICARBONATE ON VARIOUS ISOLATED ELECTRON TRANSPORT SYSTEMS

(After ref. 33; see Fig. 1.) Chloroplasts containing 33 μg chlorophyll/ml were illuminated in a continuously stirred reaction mixture (2 ml) containing 50 mM phosphate buffer (pH 6.8), 100 mM sodium formate, 100 mM NaCl and the indicated donor and acceptor system. These systems were: (1) $H_2O \rightarrow$ silicomolybdate; 5 μM DCMU and 25 μM SiMo. (2) $H_2O \rightarrow$ oxidized diaminodurene; 0.5 mM diaminodurene, 0.5 mM $(Fe(CN)_6)^{3+}$ and 0.5 μM DBMIB. (3) Reduced diaminodurene $\rightarrow$ methyl viologen; 50 μM methyl viologen, 0.5 mM diaminodurene, 2.0 mM sodium ascorbate and 1 μM DCMU. When silicomolybdate or oxidized diaminodurene was the electron acceptor, electron transport was observed as O_2 evolution. When methyl viologen was the acceptor, electron transport was followed as O_2 uptake. All data have been converted to μequiv/mg chlorophyll per h. Average of 3 experiments is shown. Note: The results of reaction (3) show that CO_2 is not needed for the System I reaction; those of reaction (1) show that CO_2 is not needed on the water side of System II reaction; and those of reaction (2) pinpoint the effect to be before the plastoquinone pool because DBMIB blocks electron flow beyond plastoquinone (see scissors No. (4), Fig. 1). These experiments and others discussed in the text show that the major effects of CO_2 are to aid electron flow from (a) the reduced primary electron acceptor quinone (Q^-) to the secondary two electron acceptor quinone (R); and (b) the reduced secondary electron acceptor quinone (R^{2-}) to the plastoquinone pool, the latter being the most significant one in terms of explaining the CO_2 effect on the Hill reaction in saturating light.

System	Electron transport (μequiv/mg chlorophyll per h)	
	$-HCO_3^-$	$+10$ mM HCO_3^-
(1) H_2O to silicomolybdate	117 ± 16	108 ± 17
(2) H_2O to oxidized diaminodurene	12 ± 1	90 ± 2
(3) Reduced diaminodurene to methyl viologen	662 ± 12	673 ± 16

and Barr [82] found that addition of bicarbonate inhibited by about 30–40% the DCMU-insensitive silicomolybdate reduction by Photosystem II but stimulated by only 10–15% the O_2 evolution associated with ferricyanide reduction in the presence of DBMIB. These authors suggested that a cyclic electron flow around Photosystem II exists (see also ref. 83; Cyclic II in Fig. 1). The silicomolybdate reduction, in Crane's interpretation, represents electrons taken from the cyclic II pathway; the inhibition of cyclic pathway by bicarbonate must be before the site of electron acceptance by silicomolybdate to explain the inhibition noted above. Inhibition of the cyclic pathway would release electrons for transfer to Photosystem I. While the stimulation of ferricyanide reduction by bicarbonate addition could qualitatively agree with the stimulation of electron transport from Q^- to the plastoquinone pool upon addition of bicarbonate to the bicarbonate-depleted chloroplasts, the inhibition of a cyclic electron transport around Photosystem II after addition of bicarbonate may be a still different effect of bicarbonate. As an alternative explanation, Crane and Barr suggested that a bicarbonate-controlled conformational change in the System II region of Photosystem II may control electron flow through the DCMU-sensitive pathway.

VC. Chlorophyll a fluorescence and delayed light emission: bicarbonate requirement for the electron flow from System II to the secondary acceptor and from this to the plastoquinone pool

A brief background on chlorophyll *a* fluorescence and delayed light emission will be necessary before we can appreciate the experiments discussed in this section. If a

chlorophyll *a* molecule is excited with light of appropriate wavelengths, it goes into the first excited singlet state within 10^{-15} s. The major de-excitation pathway of an excited chlorophyll *a* molecule (Chl a*) is the one in which the excitation energy is used to drive photosynthesis, i.e. this energy is transferred to the reaction center chlorophyll *a* molecule (P) where the excitation energy is converted, with almost 100% efficiency, into chemical energy: P is oxidized and an electron acceptor (e.g. Q) is reduced. A second pathway of de-excitation of Chl a* is the prompt (within approx. 1 ns) release of light (fluorescence). Fluorescence is an excellent indicator of several reactions of photosynthesis: different reactions can be monitored by manipulating the mode of excitation and the time of observation. As fluorescence is both a non-destructive and a sensitive tool, we have exploited it in the study of the site of CO_2 action in the Hill reaction [84]. At room temperature, 80–90% of chlorophyll *a* fluorescence originates in the pigment System II, and only 10–20% (or even less) originates in the pigment System I. The chlorophyll *a* fluorescence of the pigment System I appears to be insensitive to changes in the primary photochemistry. However, that of the pigment System II is highly sensitive to the chemistry of this system; it is even indirectly influenced by changes in the pigment System I. Duysens and Sweers [85] proposed the hypothesis that when Q (the electron acceptor of Photosystem II) is in the oxidized state, chlorophyll *a* fluorescence yield is low, and when Q is in the reduced state (as Q^-), chlorophyll *a* fluorescence yield is high; this information should be recalled later. (For reviews on chlorophyll *a* fluorescence, see refs. 48, 86, 87, 88 and 89.)

If P^+ and Q^-, produced by the primary photochemistry, recombine, instead of transferring their electrons and holes to appropriate intermediates of photosynthesis, then there is a certain probability that the chemical energy could be converted back into excitation energy, which may be transferred back to another chlorophyll *a* molecule producing an excited state (Chl a*). This Chl a* can thus emit light. This light emission is referred to as delayed light emission here; others use the general term luminescence for it. Although there is a small amount of delayed light emission from pigment System I, most of the delayed light emission originates in pigment System II and can also be used to monitor System II reactions. (For reviews on delayed light emission, see refs. 90–94.) Other necessary background information regarding chlorophyll *a* fluorescence (and to a lesser extent delayed light emission) will be presented during the discussion of the experiments.

Wydrzynski and Govindjee [75] provided the first 'direct' evidence that, at least, one site of the bicarbonate effect was after the electron donation sites of $MnCl_2$, NH_2OH, diphenyl carbazide and hydroquinone; these donors are suggested to donate electrons to the intermediates labelled Z_1 and Z_2 on the water side (left, bottom of Fig. 1). These authors used chloroplasts treated with high concentration of alkaline Tris to remove all oxygen evolving capacity (ref. 76; see scissors No. (1), Fig. 1). Therefore, a bicarbonate effect in these chloroplasts cannot be related to the oxygen evolution step. Tris-washed chloroplasts show no variable chlorophyll *a* fluorescence; this can be restored by the addition of various electron donors [76,95]. This is explained as follows. In normal chloroplasts, chlorophyll *a* fluorescence intensity rises to a fixed value (called the 'O' level) as soon as chloroplasts are exposed to light; however, as the time of illumination increases, fluorescence intensity increases to a maximum value (the 'P' level). The P minus O fluorescence is referred to as the variable fluorescence; the O to P rise is due to the reduction of Q to Q^-. The entire process of the reduction of all Q to Q^- takes some time because it takes time to reduce the several molecules in the plastoquinone

pool; thus, the area over the fluorescence rise curve represents the size of the plastoquinone pool. When the electron flow from the water side is blocked, as is done by Tris-washing, there is no net electron flow and System II remains in the oxidized state and there is no variable fluorescence. However, as substitute electron donors are provided, replacing H_2O, the variable chlorophyll *a* fluorescence is restored. In these Tris-washed chloroplasts with the various electron donors, the bicarbonate effect was still present on the variable fluorescence: in bicarbonate-depleted chloroplasts a rapid initial fluorescence rise was observed suggesting a slowing down in the reoxidation of Q^- by the plastoquinone pool. A comparison of the effects of various concentrations of DCMU (which blocks beyond Q^-; see scissors No. (3), Fig. 1), of heat treatment (see scissors No. (1), Fig. 1), hydroxylamine treatment (see scissors No. (2), Fig. 1), and of various concentrations of bicarbonate suggested that the absence of bicarbonate was equivalent to the addition of DCMU (i.e. blockage of electron flow on the reducing side of Photosystem II), but not of hydroxylamine or heat treatment (inhibition of electron flow on the oxidizing side at sites 2 and 1, respectively). Note that hydroxylamine, depending on its concentration, can block as well as restore electron flow.

The above conclusions of Wydrzynski and Govindjee were extended by Jursinic et al. [64]. In bicarbonate-depleted chloroplasts the amplitude of the electron spin resonance signal II_{vf} was about 40% reduced compared to the control sample, but there was no effect on the dark decay of the signal. The ESR signal II_{vf} is attributed to Z_2^+ [96–99], Z_2 being the second electron donor to the reaction center chlorophyll *P*-680; thus, the amplitude of signal II_{vf} represents the concentration of Z_2^+ and its dark decay the electron flow from the water side to Z_2^+. A change in Z_2^+ could be due to a change in concentration of *P*-680$^+$ or Z_1^+, and the absence of the CO_2 effect on the decay kinetics of Z_2^+ to the absence of CO_2 effect on the electron flow from H_2O to Z_2^+. Furthermore, Jursinic et al. [64] found no significant bicarbonate effect on the rise in chlorophyll *a* fluorescence yield in the microsecond time range after a 10-ns saturating flash. The yield was measured with a weak analytic beam. This experiment is most simply interpreted as follows (see for example refs. 100–102a). (We do, however, recognize that there may be alternate explanations.) A brief saturating flash creates P^+-680 and Q^-, but contrary to expectations when Q is in the reduced state, the chlorophyll *a* fluorescence yield is low; this is because P^+ is also suggested to be a quencher of fluorescence. As the electrons flow from Z_2 to Z_1 to P^+-680, the chlorophyll *a* fluorescence yield rises with the removal of the quencher P^+-680. Depending upon the experimental conditions, the chlorophyll *a* fluorescence rise may be limited by the electron flow from Z_2 to Z_1 or Z_1 to *P*-680. Thus, the absence of the CO_2 effect on the chlorophyll *a* fluorescence rise, after a saturating flash, is interpreted to mean that the site of CO_2 action is not located in the reduction of *P*-680$^+$; and the absence of CO_2 effect on the dark decay of ESR signal II_{vf}, as already noted above, is due to the absence of CO_2 effect on the reduction of Z_2^+ by the 'S' states involved in O_2 evolution [41,42,51,103,104]. Accordingly, there seems to be no major effect of bicarbonate depletion on reactions on the oxidizing (water) side of Photosystem II. On the basis of measurements on *P*-680, the decreased amplitude of the ESR signal II_{vf} is suggested to be due to (up to) 40% reversible inactivation of the reaction centers of Photosystem II in the absence of bicarbonate. This was confirmed by Siggel et al. [65], who showed that bicarbonate depletion caused up to 40% reduction in the absorption change at 334 nm that indicates the formation of the semiquinone anion Q^-, the reaction partner of *P*-680.

Jursinic et al. [64] also demonstrated that the absence of bicarbonate causes a several-

fold reduction in the chlorophyll *a* fluorescence yield decay, following a light flash. While the half-time of this decay was approx. 560 μs (without correction for a slower phase) in the presence of bicarbonate, it was 2.6 ms in its absence (Fig. 5). This phenomenon explained (see later discussion) the large increase in the relaxation of S_n' to S_{n+1} state observed earlier by Stemler et al. [61]. In these experiments, a 1 μs light flash creates *P*-680$^+$ Q$^-$, and almost within the flash or soon thereafter, *P*-680Q$^-$ is formed due to electron flow from Z_1; after this step, a weak analytic beam is utilized to monitor the chlorophyll *a* fluorescence yield. Since this beam cannot make significant changes in the concentration of Q$^-$, it monitors the fluorescence yield which is related to the concentration of Q$^-$ at the time of the measurement. Thus the decay of the chlorophyll *a* fluorescence yield, as measured by the above method, with time measures the conversion of Q$^-$ to Q; this step is mainly due to the electron flow from Q$^-$ to R, and thus the 5-fold effect of the absence of CO_2 on fluorescence decay, observed by Jursinic et al., is due to the effect of CO_2 on the Q to R reaction. This conclusion was confirmed by the experiments of Siggel et al. [65] who monitored the decay of Q$^-$ to Q by measuring absorbance change at 334 nm.

The above-mentioned results do not explain the 5- to 10-fold reduction of the Hill reaction in saturating continuous light after bicarbonate-depletion, observed by Stemler et al. [61] and others. The bottleneck reaction under steady-state conditions in the Hill reaction, i.e. the plastoquinone oxidation, has an approximate half-time of 20 ms at room temperature (see also refs. 105 and 105a for the bottleneck reaction in photosynthesis). Therefore, to explain the 5- to 10-fold reduction in the steady-state saturation rate, it must be suggested that the Hill reaction has been slowed down to yield a half-time of 100–200 ms for its bottleneck reaction. A 2.6–10 ms step could not be of much significance for the steady-state phenomenon.

Govindjee et al. [106] (see also a summary by Govindjee and Khanna [107]) were able to demonstrate that the major block caused by the absence of bicarbonate is between the secondary electron acceptor R (or B) [108,109] and the plastoquinone pool; this reaction

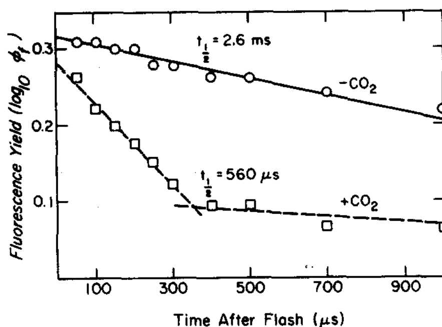
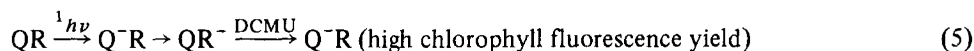
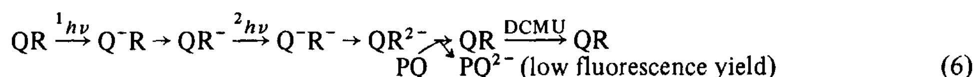


Fig. 5. Semilog plot of the decay of chlorophyll *a* prompt fluorescence yield after saturating 10-ns 337-nm pulse. Yield was measured with a variable delay analytic weak flash (Corning 4-96 filter; General Electric Strobotac 1538-A; neutral density filters). Photomultiplier, EMI 9558B protected with Wratten 2A and Schott RG-8 filters. *Lactuca sativa* chloroplasts were depleted of bicarbonate and resuspended in buffer as described by Wydrzynski and Govindjee [75]. Similar results were obtained with *Zea mays* chloroplasts. (After ref. 64.) Note: This experiment suggests that the absence of CO_2 slows down the reoxidation of the reduced form of the primary electron acceptor (Q$^-$) of pigment System II.

is slowed down to a value in the 100–200 ms range. They measured oscillations in chlorophyll *a* fluorescence due to the presence of the 2-electron acceptor R [108]. In order to appreciate the results and conclusions on the CO_2 effect, let us look at the experiments and explanations of Velthuys and Amesz [108]. In control chloroplasts, in which the electron flow leading to O_2 evolution has been blocked at site 1 (see scissors No. (1), Fig. 1) and artificial electron donors have been added which feed electrons to Z_1 or Z_2, DCMU addition induces high chlorophyll *a* fluorescence yield after an odd number of flashes, but after an even number of flashes, this yield is low. If Q^- is formed, the yield is high, but if Q is present the yield is low (see ref. 85). This can be understood by the following scheme:



$$QR \xrightarrow{^1h\nu} Q^-R \rightarrow QR^- \xrightarrow{DCMU} Q^-R \text{ (high chlorophyll fluorescence yield)} \quad (5)$$

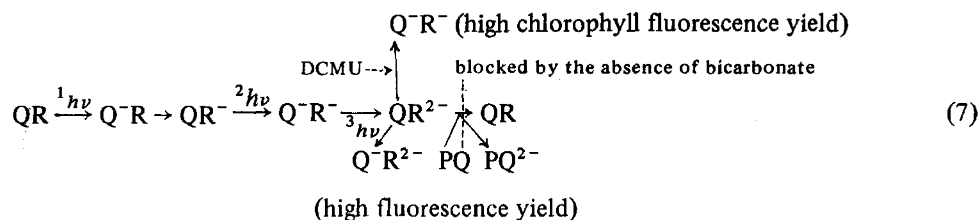


$$QR \xrightarrow{^1h\nu} Q^-R \rightarrow QR^- \xrightarrow{^2h\nu} Q^-R^- \rightarrow QR^{2-} \underset{PQ \quad PQ^{2-}}{\rightarrow} QR \xrightarrow{DCMU} QR \text{ (low fluorescence yield)} \quad (6)$$

Here, superscript on the left side of $h\nu$ (photon) refers to the number of the flash, and the other symbols have the usual meaning. In this scheme, only the reducing side of System II is shown. After one flash, QR^- is formed (Eqn. 5); it is suggested that DCMU changes the redox levels of R and Q such that the electron on R^- flows back to Q and thus chlorophyll *a* fluorescence yield, measured by a weak light beam, is high due to the presence of Q^-. On the other hand, after an even number of flashes, QR is formed (see Eqn. 6) and thus DCMU has no effect on it, and the chlorophyll *a* fluorescence yield is low due to the presence of Q. A binary oscillation is thus observed when DCMU-induced chlorophyll *a* fluorescence is plotted as a function of number of preilluminating flashes. While using hydroxylamine to block oscillations on the water side as well as to act as an electron donor, Govindjee et al. [106] could reproduce the binary oscillations in fluorescence yield upon addition of DCMU after various flashes (Fig. 6), thus confirming the data of Velthuys and Amesz [108]. The bicarbonate-depleted chloroplasts showed a complete absence of oscillations; the DCMU-induced fluorescence yield was high after all the flashes (1, 2, 3, etc.). Upon the addition of 20 mM bicarbonate the oscillations were restored (Fig. 6). The binary oscillations in the control and the CO_2-resupplied samples (Fig. 6) are explained exactly as above. If the CO_2-depletion causes a block in the QR^{2-} to QR reaction (Eqn. 6), then the addition of DCMU after the second flash would give the following reaction:

$$QR^{2-} \xrightarrow{DCMU} Q^-R^-$$

and the fluorescence yield would be high. If 3 pre-flashes were given, and QR^{2-} to QR was blocked, as suggested above, then the 3rd flash would produce Q^-R^{2-} and the fluorescence yield would be high (see Eqn. 7) and thus there would be no oscillations, as observed in Fig. 6.



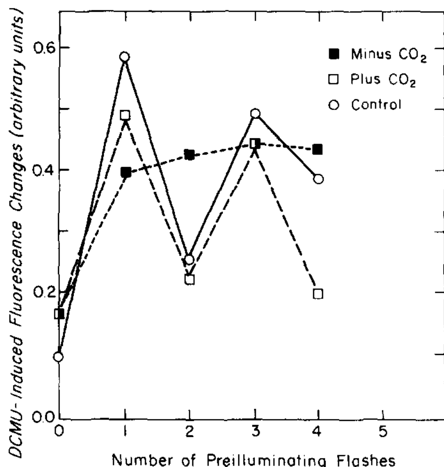


Fig. 6. DCMU-induced chlorophyll *a* fluorescence increase as a function of the number of pre-illuminating flashes. Additions as indicated. Concentrations: DCMU, 5 μM; hydroxylamine, 1 mM; bicarbonate, 20 mM; chlorophyll, 20 $\mu g \cdot ml^{-1}$ of spinach chloroplast suspension. (After ref. 106.) Note: Hydroxylamine, used in this experiment, blocked electron flow from water to P-680$^+$ and also acted as a substitute donor, thus eliminating any influence of oscillations in the 'S' states on chlorophyll *a* fluorescence. The binary oscillations, observed here, in control and CO_2-supplied chloroplasts indicate the existence of a 2-electron acceptor 'R'; the absence of oscillation in CO_2-deficient chloroplasts is explained to be due to a block in the electron flow from R^{2-} to the plastoquinone pool (see text for references and schemes).

The above results suggested an experiment in which chemicals are not added to perturb the system but the chlorophyll *a* fluorescence yield is followed after different numbers of flashes, and the long-term fluorescence yield (say, at 100–200 ms) is plotted as a function of pre-flash number. If we accept that the absence of CO_2 blocks electron flow from R^{2-} to PQ, then, after 1 flash, chlorophyll *a* fluorescence yield would be very low at 100–200 ms because by then Q^-R would all have been converted to QR^- (Eqn. 7); after 2 flashes, Q^-R^- would all have been converted to QR^{2-} and the yield would again be very low. However after 3 flashes Q^-R^{2-} would have been formed and the fluorescence yield would be high because the system would be blocked in a high fluorescence state. Successive light flashes would also give high fluorescence yield. Therefore, in order to locate the site of action of bicarbonate more precisely, long term fluorescence, after a sequence of saturating flashes, was measured. DCMU and hydroxylamine were not injected and chlorophyll fluorescence yield was measured 160 ms after each flash in control chloroplasts; the yield was independent of flash number with or without 20 mM ferricyanide (Fig. 7). In bicarbonate-depleted chloroplasts, the yield after the second flash was only slightly higher than after the first flash. However, the yields after the third and subsequent flashes were high whether ferricyanide was present or not. (The fact that ferricyanide had no effect on these and other results shows that the phenomenon under investigation had nothing to do with the electron acceptance by ferricyanide.) This result was explained by assuming a major block by bicarbonate depletion after the state Q^-R^{2-}, since this state is reached after 3 turnovers (see Eqn. 7). These are also the first experiments which show that a capacity to store 3 electrons exists before the plastoquinone pool.

The kinetics of the slow decay, plotted as the difference between the decay after the

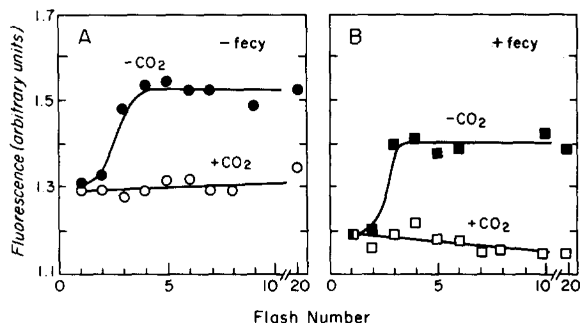


Fig. 7. Chlorophyll *a* fluorescence intensity 160 ms after the last of a series of 3-μs saturating flashes, spaced at 30 ms, as a function of the number of flashes. Additions as indicated. Concentrations: bicarbonate, 20 mM; chlorophyll, 20 μg · ml^{-1} of spinach chloroplast suspension; ferricyanide (fecy), 20 mM. (After ref. 106.) Note: This experiment shows that, in the absence of CO_2, after the third and succeeding flashes chlorophyll *a* fluorescence yield is high at 160 ms because the system is blocked in the Q^-R^{2-} state (Q being the 'primary' electron acceptor and R being the secondary two electron acceptor). (See text for references and schemes.)

third and the second flash in bicarbonate-depleted chloroplasts is shown in Fig. 8. Fluorescence decay was slow in bicarbonate-depleted chloroplasts, with a half-time in the 120–160 ms range! In view of the above explanations, this is the half time for the reduction of PQ by R^{2-} in CO_2-depleted chloroplasts. In control and bicarbonate-resupplied samples there was no significant decay in the 100–500 ms range, the fluorescence yield was very low, as the electron flow from R^{2-} to PQ is over within 1 or 2 ms.

It is evident from the above results that the block by bicarbonate depletion is after the third flash, i.e. in the state Q^-R^{2-}, and that in the absence of bicarbonate the Q^-R^{2-} decays with a half-time of 100–200 ms. Siggel et al. [65] measured absorption changes at 265 nm (due to plastoquinone) in long flashes and showed that the decay of this change is about 20 ms in control and bicarbonate-resupplied chloroplasts, while it is about 100 ms in the bicarbonate-depleted samples (Fig. 9). In control chloroplasts, this decay is a measure of plastoquinone reoxidation, and in bicarbonate-depleted samples, this decay is a measure of plastoquinone reoxidation, and in bicarbonate-depleted samples, this decay must be limited by the reoxidation of R^{2-} (R is a special plastoquinone,

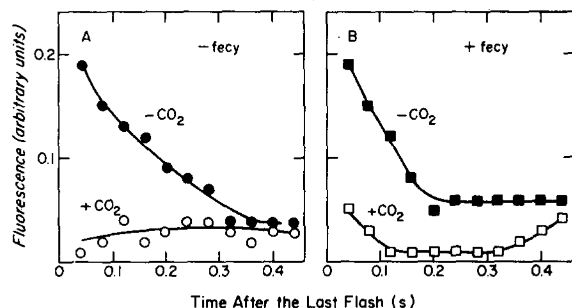


Fig. 8. Chlorophyll *a* fluorescence intensity after the third flash minus that after the second flash as a function of time after the last flash. Additions as indicated; see legend of Fig. 7. (After ref. 106.) Note: This experiment is suggested to provide the time for the decay of R^{2-} state (i.e. time for the electron flow from R^{2-} to the plastoquinone pool; it is of the order of 100–200 ms) in the CO_2-depleted chloroplasts.

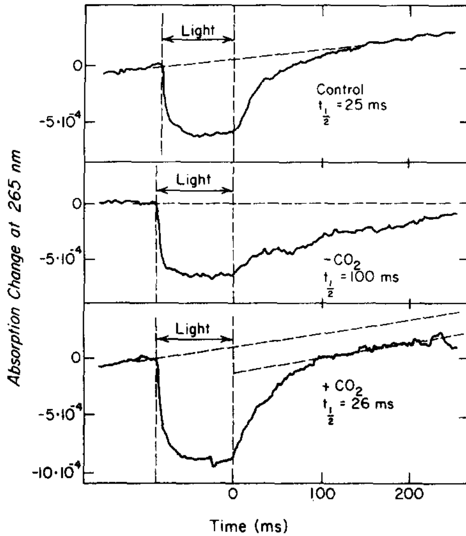


Fig. 9. Time course of the absorption change at 265 nm induced by 85 ms repetitive flashes (indicating mainly R^{2-} and PQ^{2-}, both are plastoquinones) for untreated (control), CO_2-depleted ($-CO_2$) and reconstituted ($+CO_2$) chloroplasts. The amplitudes $\Delta I/I$ are $6.8 \cdot 10^{-4}$, $6.5 \cdot 10^{-4}$, and $9 \cdot 10^{-4}$, respectively. The half-times of dark relaxation are shown. Number of flashes, 64; dark time (t_d) = 5 s; electrical bandwidth, 600 Hz. 720 nm background light ($\Delta\lambda$ = 15 nm) of 400 ergs · cm^{-2} · s^{-1} intensity was used except in the case of the control (leading to a reduced amplitude in this case). (After ref. 65.) Note: This experiment is interpreted to mean that the absence of CO_2 slows down the rate of electron flow from R^{2-} to the plastoquinone pool, and that this is the major effect of the CO_2-depletion.

distinct from that in the pool, but also having absorption changes in the ultraviolet region [110]) in view of the explanations given above.

Thus it is clear that bicarbonate depletion creates a new bottleneck reaction, with a half-time of about 100–200 ms, in the reduction of the plastoquinone pool by R^{2-}. Addition of bicarbonate relieves this effect and the normal bottleneck reaction, with a half-time of about 20 ms, in the reoxidation of reduced plastoquinone begins to be important. This therefore explains the 5- to 10-fold stimulation observed in the Hill reaction under saturating continuous light, upon addition of CO_2 to CO_2-depleted chloroplasts.

In view of the above data and conclusions, it is now possible to reinterpret the earlier results. Stemler and Govindjee [111] measured chlorophyll *a* fluorescence transients in continuous illumination and long-term 1- to 10-s delayed light emission in bicarbonate-depleted chloroplasts and those reconstituted with bicarbonate. At the time of the presentation of those data, the authors attempted to explain, without success, their results in terms of an effect on the oxygen-evolving side of electron flow. However, these results must now be reinterpreted in view of the new data which show clearly that the CO_2 effect is on the reducing side of Photosystem II.

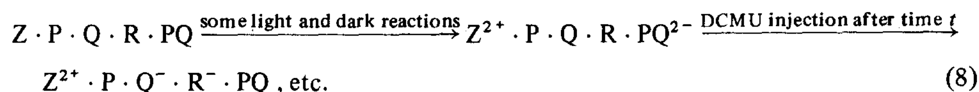
Earlier, we mentioned that upon illumination of dark-adapted chloroplasts, chlorophyll *a* fluorescence intensity increases with time of illumination from an 'O' level to a 'P' level registering the reduction of Q to Q^-. One detail was left out. First, there is a faster rise in fluorescence yield from the 'O' level to an 'I' level. Here, after 'I', there may be a brief plateau or even a dip in the yield, and then an increase to the 'P' level [48,86]. The slight slowing down of the rise in the yield following the 'I' level is due to interaction

with pigment System I which transiently converts Q^- to Q by pulling the electron away from Q^-. Bicarbonate depletion caused a faster initial rise ($O \rightarrow I$) in fluorescence intensity followed by a slower rise phase ($I \rightarrow P$) than in the control chloroplasts. Addition of bicarbonate delayed the $O \rightarrow I$ phase, but accelerated the $I \rightarrow P$ phase. The rapid increase in $O \rightarrow I$ in CO_2-depleted samples is explained by a decrease in the electron flow from Q^- to the plastoquinone pool. If the block were complete, the minus bicarbonate curve would have been like the DCMU curve, but it was not, probably due to the incomplete removal of bicarbonate. (DCMU is known to block electron flow from Q^- to the plastoquinone pool [85,112] and in its presence the fluorescence rises rapidly to the maximum value and stays high.) The slow $I \rightarrow P$ rise is due to the slow reduction of the entire plastoquinone pool. If enough time is given, the plastoquinone pool would ultimately get filled up because the block is not complete. This is shown by the finding that the intensity of fluorescence at the P level is insensitive to bicarbonate. At low light intensity, and in the presence of DCMU, which causes a complete block in electron flow from Q^- to the plastoquinone pool, there is no bicarbonate effect on the fluorescence transient.

Addition of bicarbonate to CO_2-depleted chloroplasts led to a 2- or 3-fold increase in the 1-s delayed light emission, measured after 10 or 60 s preillumination. This stimulation of delayed light by CO_2 addition can be explained by the finding that CO_2 reactivates the reaction centers partially (up to 40%) inactivated by CO_2-depletion [64], and, that the delayed light emission requires active reaction centers. The increase in delayed light emission by bicarbonate addition to CO_2-depleted chloroplasts is higher when DCMU is present. In the presence of DCMU, electron flow is blocked after Q, causing the concentration of Q^- to be higher than in the absence of DCMU; thus, the CO_2 effect on delayed light emission is larger, also because the activation of the forward reaction is of no consequence here.

It was also shown by Stemler and Govindjee [111] that the injection of DCMU gave a delayed light emission burst in normal, but not in the bicarbonate-depleted chloroplasts. This can be explained as follows. In CO_2-sufficient chloroplasts, injection of DCMU causes an accumulation of Q^-, which leads to high chlorophyll *a* fluorescence (Eqn. 8) and high delayed light emission (Eqn. 9) (P is the reaction center (*P*-680), R is the secondary acceptor quinone and PQ is the plastoquinone pool):

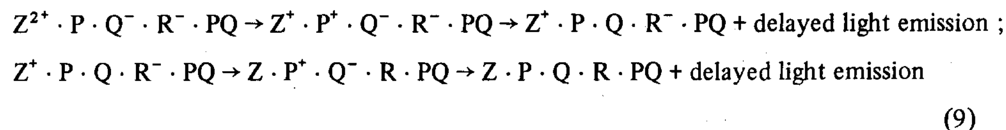
Example:



$$Z \cdot P \cdot Q \cdot R \cdot PQ \xrightarrow{\text{some light and dark reactions}} Z^{2+} \cdot P \cdot Q \cdot R \cdot PQ^{2-} \xrightarrow{\text{DCMU injection after time } t} Z^{2+} \cdot P \cdot Q^- \cdot R^- \cdot PQ\text{, etc.} \quad (8)$$

Here, chlorophyll *a* fluorescence yield is high right after DCMU addition because of the presence of the PQ^- state.

After DCMU injection after time *t*, there is also some probability of the following reactions:



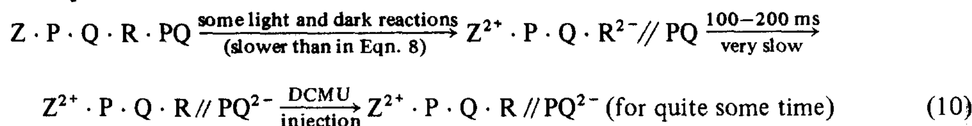
$$Z^{2+} \cdot P \cdot Q^- \cdot R^- \cdot PQ \rightarrow Z^+ \cdot P^+ \cdot Q^- \cdot R^- \cdot PQ \rightarrow Z^+ \cdot P \cdot Q \cdot R^- \cdot PQ + \text{delayed light emission};$$

$$Z^+ \cdot P \cdot Q \cdot R^- \cdot PQ \rightarrow Z \cdot P^+ \cdot Q^- \cdot R \cdot PQ \rightarrow Z \cdot P \cdot Q \cdot R \cdot PQ + \text{delayed light emission} \quad (9)$$

In the bicarbonate-depleted chloroplasts, electron flow from Q to the plastoquinone pool

is very slow (see earlier discussion); the electron flow from Q^- to the plastoquinone pool is mediated by the 2-electron acceptor quinone R (or B) [108,109] as noted above; the bicarbonate depletion slows down electron flow from R^{2-} to the plastoquinone pool (PQ) [106]; we suggest that CO_2 depletion also slows down the back flow of electrons from PQ^{2-} to R.

Example:

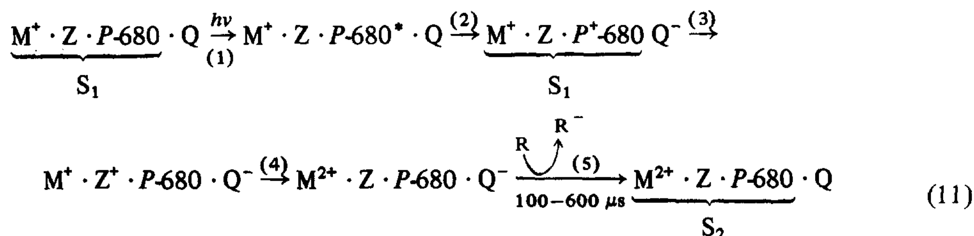


$$Z \cdot P \cdot Q \cdot R \cdot PQ \xrightarrow[\text{(slower than in Eqn. 8)}]{\text{some light and dark reactions}} Z^{2+} \cdot P \cdot Q \cdot R^{2-} // PQ \xrightarrow[\text{very slow}]{100-200 \text{ ms}}$$

$$Z^{2+} \cdot P \cdot Q \cdot R // PQ^{2-} \xrightarrow[\text{injection}]{\text{DCMU}} Z^{2+} \cdot P \cdot Q \cdot R // PQ^{2-} \text{ (for quite some time)} \qquad (10)$$

Thus, in CO_2-depleted chloroplasts, DCMU injection does not immediately increase the concentration of Q^-, a precursor of delayed light emission, and thus there is no delayed light emission burst immediately after DCMU injection.

Stemler et al. [61] (see also ref. 113) studied the effect of bicarbonate-depletion on oxygen evolution in flashing light in chloroplast fragments. The rate of the dark relaxation of the S states ($S'_n \rightarrow S_{n+1}$, where S refers to the oxidation state of the oxygen evolving mechanism [41,42,114], and n = 0, 1, or 2) was retarded in bicarbonate-depleted chloroplasts compared to its rate in depleted chloroplasts resupplied with bicarbonate. The half-time for this relaxation reaction for the various steps ($S'_1 \rightarrow S_2$, $S'_2 \rightarrow S_3$, etc.) was changed from a normal value of about 600 μs to 10 ms. This needs further explanation and background information. In order to explain the amount of O_2 evolved/flash as a function of flash number, it had been suggested that a charge accumulator exists, i.e. 4 light reactions in Photosystem II lead to accumulation of four positive equivalents on a state 'S' (or M, in Fig. 1), which in its most oxidized form S_4 reacts with 2 molecules of water to yield $O_2 + 4H^+$ and S_0 [41,42]. Furthermore, in dark-adapted chloroplasts, the ratio of $[S_1]$ to $[S_0]$ is approx. 3 : 1. Thus, only 3 light flashes, separated by appropriate dark times needed for the dark relaxation of the S states, are required to give the first O_2 burst, and, thereafter, there is a periodicity of 4 in the O_2/flash as a function of flash number. The steps involved in the conversion of S_n to S_{n+1}, e.g. S_1 to S_2, may be written as follows (we shall use Z for both Z_1 and Z_2 and M for the charge accumulator):



$$\underbrace{M^+ \cdot Z \cdot P\text{-}680}_{S_1} \cdot Q \underset{(1)}{\xrightarrow{h\nu}} M^+ \cdot Z \cdot P\text{-}680^* \cdot Q \xrightarrow{(2)} \underbrace{M^+ \cdot Z \cdot P^+\text{-}680}_{S_1} Q^- \xrightarrow{(3)}$$

$$M^+ \cdot Z^+ \cdot P\text{-}680 \cdot Q^- \xrightarrow{(4)} M^{2+} \cdot Z \cdot P\text{-}680 \cdot Q^- \xrightarrow[100-600\ \mu s]{R \curvearrowright R^-\ (5)} \underbrace{M^{2+} \cdot Z \cdot P\text{-}680}_{S_2} \cdot Q \qquad (11)$$

Stemler et al. [61] had observed, as noted above, that the relaxation of S'_1 to S_2 was changed from a half-time of approx. 600 μs to 10 ms by CO_2 depletion, but it was not clear whether reactions (3) and (4) or (5) were affected. The Q^- to Q reaction (the last step in the above series of reactions), as measured by chlorophyll *a* fluorescence decay, has been shown to range from 90 to 600 μs [64,115] in normal chloroplasts; the same half-times have been inferred by measuring absorbance changes at 320 nm [116]. The experiments of Jursinic et al. [64] on chlorophyll *a* fluorescence decay and of Siggel et

al. [65] on the conversion of $X320^-$ ($\equiv Q^-$) to X-320 indeed show that bicarbonate depletion causes retardation of Q^- to Q decay from a half-time of about 500 μs to about 3–7 ms. These results suggest that increases in half-times of $S'_n \rightarrow S_{n+1}$, observed by Stemler et al. [61,113], are indeed due to the retardation of Q^- to Q decay. Thus, the effect of bicarbonate seems to be on the Photosystem I side of Photosystem II, rather than on the water side (see middle, Fig. 1). No effect of bicarbonate on the deactivation of the S states or on the last step of oxygen evolution was documented [61,113]. However, a second effect of bicarbonate already mentioned above [61,64,65], a reversible inactivation of some of the reaction centers of Photosystem II, was proposed to explain the bicarbonate effect at low light intensities.

Since CO_2-depletion affects chlorophyll *a* fluorescence, it was considered necessary to check whether CO_2-depletion causes a change in excitation energy transfer from Photosystem II to Photosystem I. At 77 K, fluorescence emission spectra show 3 emission bands at 685, 695, 730 nm, labelled as F685, F695 and F730, respectively; the two shorter wavelength bands originate mainly in Pigment System II and F730 mainly in Pigment System I by direct absorption or energy transfer from System II (see for example refs. 117–121). If the absence of bicarbonate would decrease or increase excitation energy transfer from Photosystem II to Photosystem I, we should observe a decrease or increase in the ratio F730/F685. However, bicarbonate depletion does not change excitation energy transfer from Photosystem II to Photosystem I because the fluorescence emission spectra both at 77 K and at room temperature were the same in the presence and in the absence of bicarbonate (data of T. Wydrzynski and Govindjee, presented in ref. 84).

VD. Bicarbonate binding is close to the site where the herbicide DCMU binds

While the major site of inhibition by bicarbonate depletion seems to be well established now, almost nothing is known about the mechanism of this action. Binding of bicarbonate to chloroplasts could yield information on this mechanism of action. Such a study was undertaken by Stemler [122].

Stemler and Govindjee [31] already found that incubation of chloroplasts with bicarbonate in the dark is a prior condition for activation of oxygen evolution during ferricyanide Hill reaction. Stemler (personal communication) has recently shown that bicarbonate binds to the thylakoids only when R is in the oxidized state but not when it is in the R^- or R^{2-} state. Since the binding site of the herbicide DCMU is close to the binding site of bicarbonate (see below), this finding is consistent with the suggestion by van Rensen [123] that DCMU affects the oxidized state of a substance 'X', which is very close to, or might even be identical with the primary electron acceptor Q or Photosystem II. (At that time (1969), X was suggested to be a plastoquinone, but, since R also is a quinone [110], X is equivalent to R.)

Two binding sites for bicarbonate appear to be present in chloroplasts [122]:

(1) A very large pool of bicarbonate may exist at a concentration approaching that of the bulk chlorophyll. This may be bound to a site having a very low affinity for bicarbonate. According to Stemler (personal communication), this low-affinity pool may only be a loose association of bicarbonate on the thylakoid membrane and it appears that it does not influence Photosystem II in any way.

(2) A second, much smaller, high affinity site is present at a concentration of one bicarbonate bound per 380–400 chlorophyll molecules. This seems to be the important binding site. The bicarbonate bound in this small pool does not necessarily exchange with

free bicarbonate in the light or in the dark. Removal of bicarbonate bound in this pool is only possible in special conditions: after washing in high salt medium at pH 5.0, the chloroplasts loose more than 99% of their tightly bound bicarbonate and oxygen evolution is suppressed by more than 90%. After resupplying bicarbonate the inhibition is fully reversed. Surprisingly, washing the chloroplasts with 0.2 M silicomolybdate (pH 7.0 and low salt) removes 77% of the tightly bound bicarbonate, while initial rates of oxygen evolution remain about the same as in the control samples. This is in agreement with the results of Khanna et al. [33], who showed that the silicomolybdate Hill reaction is not affected by bicarbonate depletion. As in the presence of diphenyl carbazide, where there was a good chloroplast activity even in the absence of bicarbonate [75], it seems that in the presence of silicomolybdate, there is also good chloroplast activity even when a large percentage of bicarbonate may have been removed. Although these studies do not prove that CO_2 is not needed in the normal unperturbed system, they, however, prove that without CO_2 the H_2O to silicomolybdate reaction involving water oxidation can go on at full speed. Stemler [122] noted that alkaline Tris washing, which eliminates O_2 evolution machinery, does not remove the high affinity bicarbonate pool; Wydrzynski and Govindjee [75] had already noted that such chloroplasts show a bicarbonate effect, as monitored by changes in chlorophyll *a* fluorescence transient.

When DCMU was given to the chloroplasts prior to washing with silicomolybdate the removal of bicarbonate by silicomolybdate was retarded [122]. Therefore, Stemler postulated a very close spatial relationship between the bicarbonate binding site and the site of action of DCMU, though the two substances should not compete for exactly the same binding site. In appears that DCMU 'overlays' the bound bicarbonate, protecting it from silicomolybdate attack. This proposed site of action of bicarbonate agrees well with the site near Q proposed earlier [64,75,106].

VI. Summary and concluding remarks

It is well established now that apart from acting as a substrate in the carboxylation reaction of overall photosynthesis, CO_2 (or bicarbonate) is required for the Hill reaction (Section II). Although there are suggestions that bicarbonate is the active species, this requires proof (Section III). Two effects of bicarbonate on chloroplast reactions may be distinguished:

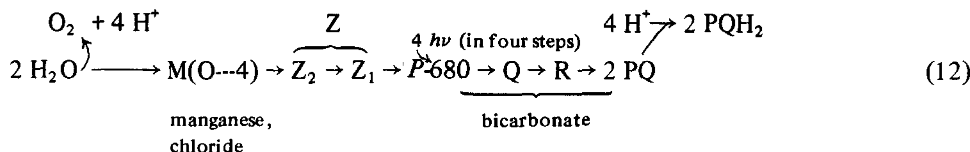
(1) a requirement for bicarbonate in photosynthetic electron transport: after depleting chloroplasts of bicarbonate, the Hill reaction is largely suppressed and is fully restored upon readdition of bicarbonate;

(2) a stimulation of photophosphorylation by addition of bicarbonate to non-depleted chloroplasts, caused by an effect, most likely, on the chloroplast coupling factor.

The site of bicarbonate action in photosynthetic electron transport seems to be well established now (Section V; Fig. 1). Absence of bicarbonate causes up to 40% inactivation of Photosystem II reaction centers (*P*-680), depending on the extent of depletion. We believe that this explains the bicarbonate stimulation of the Hill reaction at low light intensities. In addition, absence of bicarbonate causes a several-fold reduction in the rate of electron transport from Q^- (the reduced primary electron acceptor of Photosystem II) to the plastoquinone pool, with the major site between R (the secondary electron acceptor of Photosystem II, also a quinone) and the plastoquinone pool. This explains the bicarbonate stimulation of the Hill reaction at high light intensities. Since there is no

significant effect of bicarbonate on the oxidizing (water) side of Photosystem II, bicarbonate seems not to be involved in the oxygen evolution mechanism and thus is not the source of photosynthetically produced oxygen (Sections IV and V).

The path of 2 pairs of electrons from water to the plastoquinone pool can be summarized as follows (also see Fig. 1).



(12)

where, M(0---4), usually referred to as S, represents the charge accumulator [41,42,52,103,104], Z_2 and Z_1 are secondary electron donors (see for example ref. 102). Z_2^+ is responsible for ESR signal II_{vf} [99], *P*-680 is the reaction center chlorophyll *a* [124,125], Q, a quinone, is the 'primary' electron acceptor [126,127], R, also a quinone [110], is the secondary electron acceptor [106,108,109], and PQ is the plastoquinone pool [128]. The intermediate 'M' involves manganese [52] and, perhaps, requires chloride ions [53,79]. Water protons are released not all at once, but in several steps, to the interior of the thylakoid vesicle [129–131], and protons needed for the conversion of PQ^{2-} to PQH_2 are taken from the outer side of the thylakoid membrane [132].

In the above scheme, CO_2 seems to have no effect on the electron flow from H_2O to Z_2^+ [64], from Z_2 to *P*-680$^+$ [64], and from H_2O to Q [33]. However, CO_2 has about 2-fold effect on the reactivation of the reaction center chlorophyll *a* [61,64,65] explaining the slight dependence of CO_2 at low light intensities in the Hill reaction; it has about 5- to 10-fold stimulatory effect on the Q to R reaction [64,65], explaining the recovery rates of the S (or M) states [61]; and it has about 100-fold stimulatory effect on the half-time of the reduction of PQ by R^{2-} [65,106]. Whether this effect is on the *P*-680 · Q · R complex, and/or it is due to the effect of bicarbonate or CO_2 in bringing protons or even electrons to plastoquinone remains to be elucidated. It would be important to study the influence of bicarbonate-depletion on proton translocation at the plastoquinone site.

Up to now, very little is known about the mechanism of action of bicarbonate in photosynthetic electron transport. One possible mode of action, mentioned above, might be through an indirect effect on the conformation of the R complex in such a way that electrons from Q are unable to be transferred to R and from R to the plastoquinone pool. Conformational changes might be brought about by the binding of bicarbonate to the membrane components to which R is attached. Large conformation changes, however, are ruled out by the finding of Stemler and Govindjee [31] that glutaraldehyde fixed chloroplasts continue to exhibit bicarbonate dependent dichlorophenol indophenol reduction. Because glutaraldehyde is a protein 'fixation' agent, structural changes in lipids caused by bicarbonate remain possible. Renger [133], from results of experiments with trypsin treatment, suggested that the Q to R region is separated from the outside of the thylakoid membrane by a proteinaceous shield. Could it be that bicarbonate regulates electron flow in the Q-region by binding to this proteinaceous shield? It would be very rewarding to see if the bicarbonate effects is eliminated in trypsin-treated thylakoid preparations. Perhaps this proteinaceous shield is also a clue to the silicomolybdate experiment of Stemler [122]. Does silicomolybdate interfere with, or does it bypass this proteinaceous shield? Isolation of the component(s) to which radioactive bicarbonate is bound is necessary to understand the molecular mechanism of bicarbonate action. It is

important to note that the electron flow path from System II to plastoquinone probably is located within a lipid environment. While it is known that CO_2 is lipid soluble, Kreutz [134] reported that phosphatidylethanolamine possesses the quality of accomplishing temperature-independent reversible phase transitions by bicarbonate and carbonic acid binding. This needs further exploration. Papageorgiou (personal communication) suggests that if phosphatidylethanolamine is somehow involved in the CO_2-generated phenomenon, then it will be worth studying these effects with cell-free membrane preparations from *Anacystis nidulans*, whose membranes do not contain this lipid.

We draw the attention of the readers to the remark of Heise and Gaffron [20] that the dependence of the Hill reaction on CO_2 may be 'just another case among many similar effects'. Lopéz-Moratalla et al. [135] reported that, while ATPase activity in rat liver mitochondria normally is stimulated by addition of bicarbonate, this stimulation is greatly decreased in isolated mitochondria from rats subjected to a variety of metabolic conditions, such as fasting, cold exposure, or diabetes. The basal ATPase activity without addition of bicarbonate was not affected. The loss of bicarbonate sensitivity was correlated well with an increase in plasma free fatty acids. To further elucidate the mechanism of bicarbonate action in the Hill reaction, it may also be worthwhile to learn from the bicarbonate effects in these [135] and other systems (see for example ref. 136).

No effort has yet been made to answer the question: what good is this bicarbonate effect for the whole plant growing in the natural environment? We do not have the answer to this question; we would like to further ask whether bicarbonate could have a regulatory role in vivo in the electron flow and phosphorylation. We hope that further experiments will answer these questions.

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