

⊗ CHLOROPHYLL-*a* FLUORESCENCE AS A PROBE
FOR LOCATING THE SITE OF BICARBONATE ACTION
IN PHOTOSYSTEM II OF PHOTOSYNTHESIS

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By

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In addition to acting as the ultimate electron acceptor in photosynthesis, CO₂ (bicarbonate) plays another important role during the electron flow from water to the plastoquinone pool (photosystem II reactions). Bicarbonate depletion of isolated chloroplasts leads to a reversible increase in the half-time of decay of chlorophyll *a* fluorescence yield, after a brief saturating flash, from 600 μsec to 2.6 msec [1]. This is due to the slowing down of the reoxidation of Q⁻ to Q by about fivefold (where Q is the primary electron acceptor of photosystem II) and it explains the severalfold decrease in the relaxation rate of the S_n' to S_{n+1}' states of the oxygen evolving system observed by A. Stemler, G. Babcock and Govindjee [2].

Recent chlorophyll *a* experiments of Govindjee, M. P. J. Pulles, R. Govindjee, H. J. van Gorkum and L. N. M. Duysens [3] have now provided an explanation of the five-to-tenfold decrease in the rate of electron flow under continuous saturating light. In bicarbonate-depleted chloroplasts, the chlorophyll *a* fluorescence decayed with a half-time of about 150 msec after the third and subsequent flashes, but appreciably faster after the first or the second flash of a series of brief saturating light flashes, given after a dark period. In control and bicarbonate-depleted chloroplasts resupplied with 10 to 20 mM bicarbonate, the decay was fast after all the flashes of the sequence. This was interpreted to indicate that the bicarbonate depletion causes a major block in the reoxidation of the secondary acceptor (R; in its doubly reduced state R²⁻) by the plastoquinone pool. This conclusion was consistent with the measurements of the DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea)-induced chlorophyll *a* fluorescence yield increase in hydroxylamine-treated chloroplasts, after a series of light flashes in the presence and absence of bicarbonate. If the bottleneck reaction of electron flow in Hill reaction, which is about 20 msec in normal chloroplasts, is increased to about 105 msec, about eightfold reduction in the saturation rate of this reaction is predicted. It is suggested that the usual bottleneck reaction of the oxidation of plastoquinone (~20 msec) is replaced by a new bottleneck reaction of the oxidation of R²⁻ by plastoquinone pool (~150 msec) in bicarbonate-depleted chloroplasts.

Finally, chlorophyll *a* fluorescence yield rise in the microsecond range (interpreted to be due to the electron donation by Z to P680⁺) and the decay of ESR signal II_{vc} (due to the electron donation from H₂O side to Z⁺) remain unchanged by bicarbonate depletion suggesting that bicarbonate action is not located on the oxidizing side of photosystem II [1].

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1. Introduction

± Fluorescence

Both the prompt and delayed emission from photosynthesizing plants and bacteria are due to deexcitation of the first singlet excited state of chlorophyll *a* (or bacteriochlorophyll); they both have almost identical emission spectra (see, e.g. LAVOREL [4]). The measured lifetime of the prompt fluorescence is almost linearly proportional to the quantum yield (see, e.g. [5]), and is in the nanosecond range (see, e.g. MAR *et al.* [6]). The quantum yield of chlorophyll (Chl) *a* fluorescence *in vivo* is

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of the order of 0.03 and the yield of delayed emission is several orders of magnitude lower (see, e.g. STACY *et al.* [7]).

The difference between prompt (to be referred from now on simply as fluorescence) and delayed emission (to be referred to as DLE) lies in the steps that lead to the production of the excited singlet state; for DLE, it is mainly by the recombination of the primary products of the so-called system II light reactions, and for fluorescence, it is either by direct absorption of a quantum, or by deexcitation of higher excited states.

Measurements of Chl *a* fluorescence have been of a great value in the investigations of photosynthesis (see reviews by GOVINDJEE *et al.* [8]; GOVINDJEE and PAPAGEORGIOU [9]; GOEDHEER [10]; PAPAGEORGIOU [11]; LAVOREL [12]). The following discussion will rely mainly on the work done in our laboratory and citation to the work of other authors could be found in the cited reviews and our papers. These include measurements on the fluorescence excitation and emission spectra, fluorescence induction and polarization of fluorescence, providing information on the composition of pigment systems I and II [13—16], on the excitation energy transfer from various accessory pigments to Chl *a*, from one spectral form of Chl *a* to another [17—21]; among the different molecules of chlorophyll *a* [22—23] among different photosynthetic units of pigment system II [5—24], and from such units of system II to pigment system I [23, 25—27].

A working hypothesis (Fig. 1) is as follows. Functionally speaking, there is a *tetrapartite* arrangement. Two light harvesting assemblies I and II are connected to two reaction center complexes I and II which are in close proximity to each other. Two reaction center molecules are assumed to be "special pairs" of Chl *a* molecules but in different microenvironments (P700 and P680). The system II units are not only close to other system II units for excitation energy exchange, but also to other system I units to account for system II → system I transfer. The mechanism of energy transfer is not yet certain but there are indications that it is by Förster's slow transfer method; other mechanisms have not yet been excluded (see [19—23, 28]). There is obviously energy exchange among system II units (see [29]) and from system II to system I units (see, e.g. review by PAPAGEORGIOU [11]).

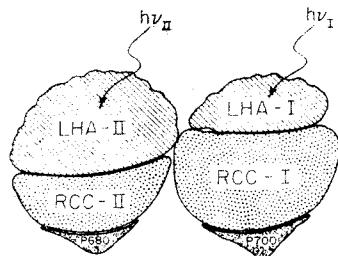


Fig. 1. A functional model for the two photosystems of photosynthesis. LHA, light-harvesting assembly; RCC, reaction center complex; P680, reaction center chlorophyll *a* of pigment system II; P 700, Reaction center chlorophyll *a* of pigment system I. There is no experimental evidence that two physically separable LHA's exist in chloroplasts. LHA I and II indicated contribution of the antenna complex to each photosystem.

The fast Chl *a* fluorescence changes in the 1 to 10 microsecond range have been used to monitor the recovery of the reaction center Chl *a* of system II (P680) from its oxidized to its reduced state (P680⁺ has been suggested to be a quencher of Chl *a* fluorescence) [30]. Chl *a* fluorescence has been used, for quite some time [31], as an indicator of the redox state of the primary electron acceptor of the system II (Q). When changes due to P680 are not significant (or not being monitored) reduction of Q leads to an increase in the Chl *a* fluorescence yield and its oxidation leads to a decrease in this yield; the decay of Chl *a* fluorescence yield in the 10 to 1.000 μsec after a saturating light flash,

perature cyt $t_{b_{559}}$ acts as an endogenous donor on this side. Another electron acceptor, separate from Q and labeled W, has recently been suggested by VAN BEST and DUYSSENS [36] to exist, from μ sec delayed light emission measurements. There is a possibility that this may be a phaeophytin molecule and serves as the real primary electron acceptor prior to Q.

2. The bicarbonate effect

In order to appreciate the use of Chl *a* fluorescence in understanding the site of bicarbonate effect in the electron flow in system II reactions, it is instructive to provide a brief review of this effect.

WARBURG and KRIPPAHL [37] discovered that CO_2 was necessary for the Hill reaction (production of O_2 with quinone as an electron acceptor) in algae. WARBURG argued that this phenomenon and the uncertainties involved in the original ^{18}O experiments of RUBEN *et al.* [38] which had led to the belief that O_2 in photosynthesis originates in H_2O must be abandoned; instead, he suggested that O_2 originated from CO_2 . The stimulation of Hill reaction by CO_2 (or bicarbonate anion) has been studied by STERN and VENNESLAND [39], IZAWA [40], GOOD [41], HEISE and GAFFRON [42], BATRA and JAGENDORF [43], VENNESLAND *et al.* [44], and WEST and HILL [45], but no explanation of this phenomenon was obtained. STEMLER and GOVINDJEE [46–48] have shown that bicarbonate is involved in system II reactions (see [49]). However, STEMLER and RADMER [50], showed that when $\text{NaH}^{18}\text{CO}_3$ is injected into bicarbonate-depleted chloroplasts, all evolved oxygen is in $^{32}\text{O}_2$, not $^{36}\text{O}_2$. WYDRZYNSKI and GOVINDJEE [51] showed that at least one site of bicarbonate effect was on the reducing side of system II.

JURSINIC *et al.* [1] demonstrated that absence of bicarbonate causes a fivefold reduction in the decay rate of the chlorophyll *a* fluorescence yield after a brief saturating flash of light: the half-time of this decay (which is due to the decay of Q^- to Q, Q being the primary electron acceptor of system II) was increased from the normal 600 μ sec to 2.6 msec (Fig. 2). This phenomenon explained the large increase in the relaxation of S_n to S_{n+1} state observed earlier by STEMLER *et al.* [2].

Bicarbonate depletion also causes a reversible inactivation of about 50% of the reaction centers of pigment system II explaining the about twofold decrease in the rate of electron flow at low light intensities, and a twofold decrease in the amplitude of the electron spin resonance signal II_{v} due to the oxidized Z (where Z is the first secondary electron donor to the reaction center chlorophyll *a* P680) [1].

The above-mentioned effects did not explain the five-to-tenfold reduction of the Hill reaction in saturating continuous light. The bottleneck reaction under steady-state conditions in the Hill reaction has an approximate half-time of 20 msec. Therefore, to explain the five-to-tenfold reduction in the steady-state saturation rate, we must suggest that the Hill reaction has been slowed down to yield a half-time of 100 to 200 msec. A 2.6 msec step could not be of much significance for the steady-state phenomenon. GOVINDJEE *et al.* [3] have now shown that the major block caused by the absence of bicarbonate is between the component R and the plastoquinone pool; and, this reaction is slowed down to a value in the 100–200 msec range.

Absence of bicarbonate in broken chloroplasts isolated from spinach led to (a) the elimination of the oscillations, with a period of two, in chlorophyll *a* fluorescence yield after a series of saturating light flashes followed by injection of $5 \mu\text{M}$

DCMU and 1 mM hydroxylamine [32]; (b) a slow decay of fluorescence yield after illumination with 4 sec continuous saturating blue light: At one second after this light was turned off, the variable fluorescence yield was severalfold higher in the absence than in the presence of bicarbonate; (c) a large increase in fluorescence yield, measured after 150 msec of the cessation of the third and the following light flashes, but not

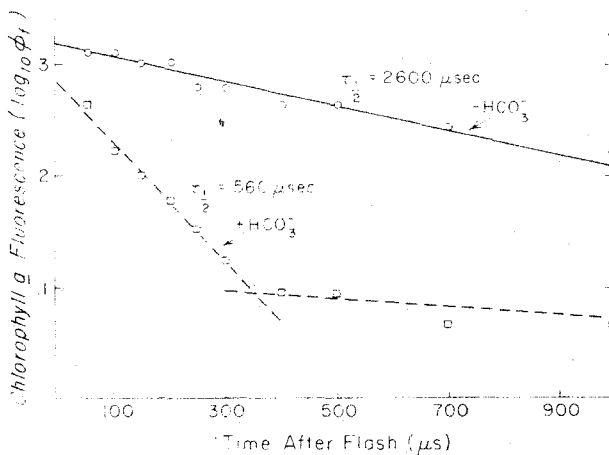


Fig. 2. Semilog plot of the decay of chlorophyll *a* fluorescence yield after saturating 10 nsec 337 nm pulse. Yield was measured with a variable delay analytic weak flash (Corning 4—96 filters; General Electric Strobotac 1538—A; neutral density filters). Photomultiplier, EMI 9558B protected with Wratten 2A and Schott RG—8 filters. *Lactuca sativa* chloroplasts were depleted of bicarbonate and resuspended in buffer as described by Wydrzynski and Govindjee [51]. Similar results were obtained with *Zea mays* chloroplasts. (After Jursinic *et al.* [1].)

after the first and second flashes; and (d) a large difference in the fluorescence yield after the third minus the second flash—this difference had an approximate half-time of 150 msec. Addition of 10 to 20 mM bicarbonate to bicarbonate-depleted chloroplasts, at about pH 7.0, restored the conditions prevailing in the untreated controls. The above results have been explained by proposing that the major block in electron flow is between the component R and the plastiquinone pool to yield a rate limiting step in the range of 100 to 200 msec. This then is the explanation of Warburg phenomena in saturating light.

Slow fluorescence decay after 4 sec saturating light

JURSINIC *et al.* [1] measured the Chl *a* fluorescence decay after a brief saturating flash and showed that absence of bicarbonate caused a fivefold slowing down of the decay rate of P to Q in the 0.05 to 2 msec range. This range was not enough to uncover changes in the longer time region needed to explain the steady-state effects. Fig. 3 shows the decay of chlorophyll *a* fluorescence up to 3 secs. Chloroplasts with

5 μM DCMU, bicarbonate-depleted samples with 2 mM ferricyanide, bicarbonate-depleted samples resupplied with 10 or 20 mM NaHCO_3 and 2 mM ferricyanide, and control chloroplasts with 2 mM ferricyanide were exposed to a saturating pulse (4 sec) of blue light. The decay of fluorescence yield was measured after the cessation of illumination with weak flashes. The minus HCO_3^- samples showed a decay curve intermediate between DCMU and control or plus HCO_3^- samples. At about 1 sec after saturating light was turned off, the yield of the "variable fluorescence" was still 50% of that in the DCMU case, whereas in control chloroplasts, there was no "variable" fluorescence; the ratio of the yield of variable fluorescence in minus HCO_3^- plus HCO_3^- was in the range of 10–15 in several experiments. This experiment shows that bicarbonate-depleted chloroplasts are blocked in a high fluorescence state even up to 1 sec suggesting that certain reactions are slowed down beyond the msec region as suggested earlier. In order to find the site of this slow reaction, we looked at the reactions associated with the component "R" which is between Q and the PQ pool [3].

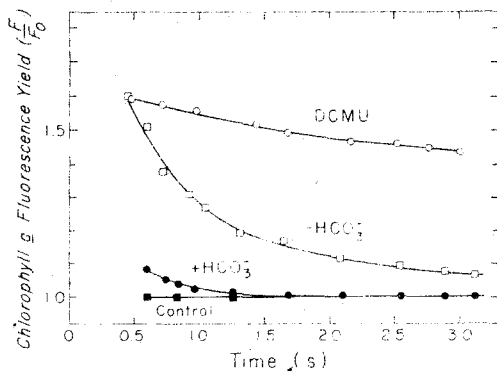


Fig. 3. Ratio of chlorophyll *a* fluorescence intensity (F) to the "0" level fluorescence (F_0) as a function of time (t) after cessation of 4 sec strong blue light illumination. Control, spinach chloroplasts suspended in phosphate buffer, pH 6.8; DCMU, control chloroplasts treated with $10 \mu\text{M}$ DCMU. Minus HCO_3^- , bicarbonate depleted chloroplasts resupplied with 20 mM HCO_3^- .

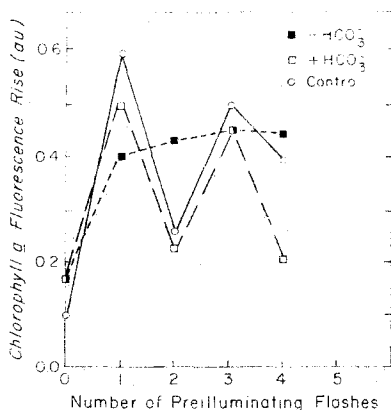


Fig. 4. 3-(3,4-dichlorophenyl)-1,1 dimethylurea induced chlorophyll *a* fluorescence increase as a function of the number of preilluminating flashes. Additions as indicated. Concentrations: DCMU, $5 \mu\text{M}$; hydroxylamine, 1 mM; bicarbonate, 20 mM; Chl, $20 \mu\text{g ml}^{-1}$ of spinach chloroplast suspension. (After Govindjee *et al.* [3])

Oscillations in chlorophyll *a* fluorescence due to "R"

If the absence of HCO_3^- blocks or slows down the QR^{2-} to QR reaction (see Eq. 2(b)), then QR^{2-} would accumulate and the following reaction would occur upon DCMU injection: $\text{QR}^{2-} \xrightarrow{\text{DCMU}} \text{Q}^- \text{R}^-$, and the fluorescence will be high as Q^- is produced; this could, however, decay at long times. Subsequent flash could produce $\text{Q}^- \text{R}^{2-}$ and the fluorescence will remain high. (This point is further tested in the next section.)

* Plus HCO_3^- , bicarbonate-depleted chloroplasts resupplied with 20 mM HCO_3^- .

Fig. 4 shows our results (see GOVINDJEE *et al.* [3]) on DCMU-induced chlorophyll *a* fluorescence rise under the above conditions. The open-circled curve shows oscillations in control chloroplasts confirming the data of VELTHUYS and AMESZ [32]; here, 1 mM hydroxylamine was used to block oscillations on the water side as well as to act as an electron donor. The minus HCO_3^- sample (solid squares) showed a complete absence of oscillations in three experiments we performed. Addition of 20 mM HCO_3^- was enough to restore the oscillations (open squares). Thus, these data are consistent with a block in the QR^{2-} to QR reaction. In order to further locate the site of HCO_3^- action more precisely, we measured long term fluorescence (100 to 500 msec) after a sequence of saturating flashes as described below.

Fluorescence yield after a series of flashes

If DCMU and hydroxylamine were not injected and chlorophyll *a* fluorescence yield was measured 150 msec after each flash (1 through 20), the yield was independent of flash number with and without 2 mM ferricyanide (Figs. 5A and 5B). However, in minus bicarbonate samples, the yield after the second flash was only slightly higher than after the first flash. But, the yields after the third and subsequent flashes were high whether ferricyanide was present or not. Data for minus bicarbonate samples was interpreted by the following hypothesis. After the first flash, the following reactions occur: $\text{QR} \xrightarrow{h\nu} \text{Q}^-\text{R}$ and Q^- decays to Q with a half-time of 2.6 msec (see

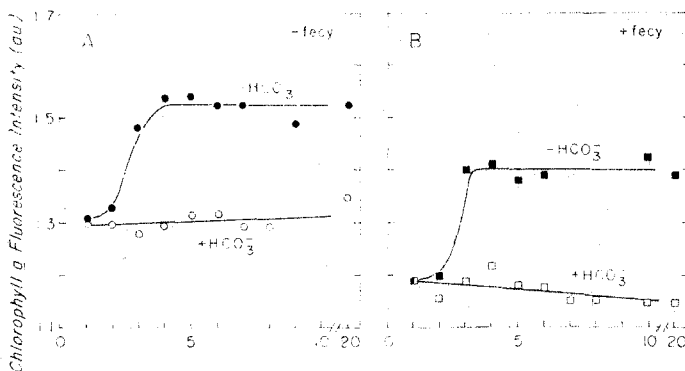


Fig. 5. Chlorophyll *a* fluorescence intensity 160 msec after the last of a series of 3 μs saturating flashes, spaced at 30 msec, as a function of the number of flashes. Addition as indicated. Concentrations: Bicarbonate, 20 mM; Chl, 20 $\mu\text{g ml}^{-1}$ of spinach chloroplast suspension. Ferricyanide, 20 mM. (After Govindjee *et al.*, 1976.)³

[1]. After two flashes $\text{QR} \xrightarrow{h\nu} \text{Q}^-\text{R} \rightarrow \text{QR}^- \xrightarrow{h\nu} \text{Q}^-\text{R}^- \rightarrow \text{QR}^{2-}$ reactions occur and the fluorescence has decayed away with a half-time of 2.6 msec (last step in the above scheme). However, after the third flash:

$\text{QR} \xrightarrow{h\nu} \text{Q}^-\text{R} \rightarrow \text{QR}^- \xrightarrow{h\nu} \text{Q}^-\text{R}^- \rightarrow \text{QR}^{2-} \xrightarrow{h\nu} \text{Q}^-\text{R}^{2-}$ reactions occur and the fluorescence decays with a long half-time because of the block beyond this step, *i.e.* from $\text{Q}^-\text{R}^{2-} \rightarrow \text{Q}^-\text{R}$. Subsequent flashes, given after 30 msec, produce

$$\text{PQ} \xrightarrow{\text{PQ}^{2-}}$$

high fluorescence as the system is blocked, in this time scale, in the Q^-R^2 -step and no further reaction can occur. Thus, these experiments show that the major block is between R and the PQ pool.

Fig. 6A and 6B show the fluorescence decay after the third flash minus that after the second flash. It is clearly shown that the fluorescence decay is slow in minus-

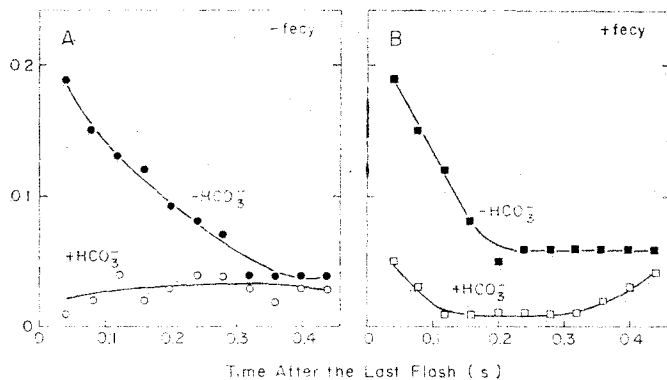


Fig. 6. Chlorophyll *a* fluorescence intensity after the third *minus* that after the second flash, as a function of time. Additions as indicated; see legend of Fig. 5. (After Govindjee *et al.* [3].)

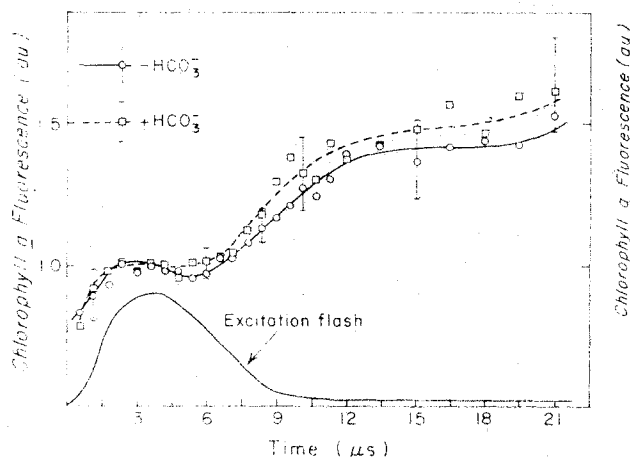


Fig. 7. Rise in chlorophyll *a* fluorescence yield during and after an excitation flash with and without 10 mM bicarbonate, normalized at $\tau=3$ usec. A trace of excitation flash intensity as a function of time is also shown. Excitation light flashes were provided by a General Radio Strobotac 1538—A through two Corning C. S. 4—96 filters. An EMI 9558 B photomultiplier protected by neutral density filters, a C. S. 2—64 and a Schott RG—8 filter combination was used. *Lactuca sativa* chloroplasts treated as described by Wydrzynski and Govindjee [51]. Similar results were obtained with *Zea mays* chloroplasts. (After Jursinic *et al.* [1].)

bicarbonate samples; in plus-bicarbonate and control samples, there is no significant decay in the 100—500 msec range—the fluorescence yield is very low. At about 150 msec after the flash, the yield of fluorescence is about ten times higher in minus-compared to plus-bicarbonate samples.

It is evident from the above experiments that after the third flash, the block is a major one and Q^-R^{2-} decays with a half-time in the range of 100—200 msec. It was not possible to measure the exact time of this reaction here. However, unpublished experiments of R. KHANNA, GOVINDJEE, U. SIGGEE, and G. RENGE on absorption changes at 265 nm, made in long flashes, show that the decay of this change is 20 msec in control and plus-bicarbonate samples, whereas it is 100—150 msec in the minus-bicarbonate samples. In control chloroplasts, this decay is a measure of plastiquinone reoxidation, and in minus-bicarbonate samples, this decay is a measure of R^{2-} reoxidation as both "R" and PQ are quinones (M. P. J. PULLES, personal communication).

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Fluorescence rise in microsecond region

Chlorophyll *a* fluorescence yield rise in the microsecond range (interpreted to be due to the electron donation by Z to $P680^+$) remained unchanged by bicarbonate depletion (see Fig. 7) suggesting that bicarbonate action is not located on the oxidizing side of photosystem II [1].

Fluorescence spectra at room and low temperatures

Fluorescence spectra at 77 K show three major bands at 685 (F685), 695 (F695) and 730 nm (F730). F685 and F695 originate mainly in pigment system II and F730 in pigment system I (see, e.g. GASANOV and GOVINDJEE [16]). If there is a change in excitation energy transfer from pigment system II to I, it is reflected in a change in the ratio of F685 + F695 to F730. The ratio of F685 + F695/F730 was the same in bicarbonate depleted and depleted chloroplasts resupplied with 10 or 20 mM HCO_3^- . Fig. 8B (after T. WYDRZYNSKI and GOVINDJEE, unpublished observations) shows the ratio of emission spectra of plus to minus HCO_3^- samples showing the absence of change in excitation energy transfer from pigment system II to I. The same results were obtained at room temperature (see Fig. 8A) where F685 is mostly from system II and emission in the 715 nm region has a relatively stronger contribution from pigment system I.

3. Concluding remarks

Our experiments have now established that the bottleneck reaction in minus-bicarbonate sample is about 150 msec and is due to slow reduction of PQ by R^{2-} . These data explain, for the first time, the inhibitory effect of the absence of HCO_3^- on the steady-state Hill reaction in saturating continuous light. Thus, measurements on Chlorophyll *a* fluorescence have yielded information on the major site of action of bicarbonate on the electron flow in system II reactions. In addition, fluorescence experiments have also shown that bicarbonate does not affect electron flow from Z to

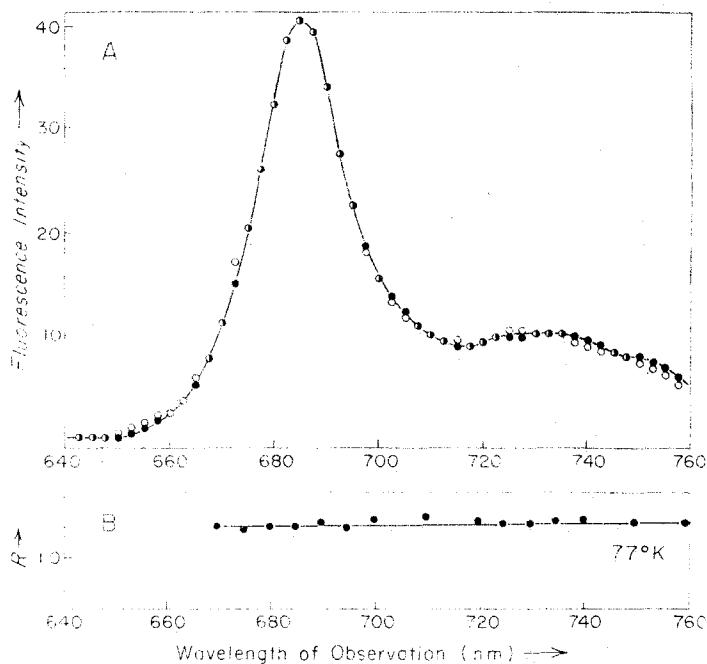
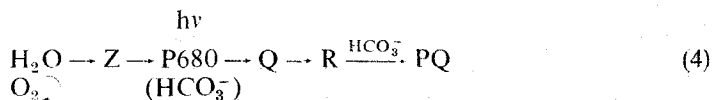


Fig. 8. (A). Emission spectra at room temperature under anaerobic conditions (N_2 atmosphere). ●, minus HCO_3^- ; ○, plus 10 mM HCO_3^- . Curves normalized at 685 nm; average of two spectra for each treatment; λ excitation, 435 nm with 24 nm slits, and Corning 4-96 filter; observation, 3.3 nm slit width; Corning C. S. 2-58 filter before the monochromator. Photomultiplier, EMI 9558B; [Chl], $10 \mu g \text{ ml}^{-1}$ of *Zea mays* chloroplast suspension. Assay medium, 0.05 M phosphate buffer, pH 6.8; 0.25 M NaCl; 0.04 M Na acetate. Same results were obtained with *Lactuca sativa* chloroplasts. (After T. Wydrzynski and Govindjee, unpublished observations of 1973.)

(B). Ratio of relative fluorescence yield at 77 K between plus HCO_3^- and minus HCO_3^- samples as a function of wavelength of observation. Conditions same as in (A) above; samples were frozen in dark under N_2 atmosphere. (After Wydrzynski and Govindjee, unpublished observations)

P680, and that it does not change the spillover of excitation energy from pigment system II to I. The slowing down of electron flow from Q^- to R may only be a consequence of block between R and PQ. In brief, we may write system II reaction as follows:



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ОПРЕДЕЛЕНИЕ МЕСТА ДЕЙСТВИЯ CO_2 ВО II-ОЙ ПИГМЕНТНОЙ СИСТЕМЕ ФОТОСИНТЕЗА С ПОМОЩЬЮ ФЛУОРЕСЦЕНЦИИ ХЛОРОФИЛЛА-*a*

Говиндjee

Кратковременное (600 мсек — 2,6 мсек) освещение хлоропластов при низком содержании CO_2 время жизни флуоресценции хлорофилла-*a* увеличивается по сравнению с не обработанной пробой. Это объясняется тем, что реокисление Q^- в Q приблизительно в пять раз уменьшается после обработки. Кроме этого ингибируется реокисление вторичного акцептора электрона (R^2^-) при лишении CO_2 .

По нашему мнению действие CO_2 объясняется не только влиянием на окислительную сторону II пигментной системы, так как после удаления CO_2 выход флуоресценции хлорофилла-*a* увеличивается в микросекундной области времени ($Z^- + P680^+ \longrightarrow Z + P680$) и сигнал ЭПР Π_{vr} (перенос энергии от H_2O на Z^+) не изменяется.