EFFECTS OF BICARBONATE ION ON CHLOROPHYLL *a* FLUORESCENCE TRANSIENTS AND DELAYED LIGHT EMISSION FROM MAIZE CHLOROPLASTS

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Abstract—Using high-intensity actinic light, the chlorophyll *a* fluorescence transient from HCO_3^--depleted chloroplasts shows a rapid initial rise (O → I) followed by a slow phase (I → P). In the presence of HCO_3^-, the O → I rise is delayed but the I → P phase is much more rapid. Using low-intensity actinic light, the chlorophyll *a* fluorescence transient from 3-(3,4-dichlorophenyl)-1,1 dimethylurea (DCMU)-treated chloroplasts is delayed in the presence of HCO_3^-. Bicarbonate increases the amount of delayed light emission from chloroplasts given 10 s illumination with weak blue light (0·4 W/m²). DCMU greatly increases the amount of delayed light seen in the presence of HCO_3^- under these conditions but decreases the amount seen in the absence of HCO_3^-. It is suggested that HCO_3^- may somehow form or stabilize, in the dark, a number of reaction centers corresponding to the S_1 state in the model of B. Forbush, B. Kok and M. McGloin (*Photochem. Photobiol.* **14**, 307–321, 1971).

INTRODUCTION

The ability of CO_2 to stimulate the Hill reaction effected by isolated chloroplasts was first clearly demonstrated by Warburg and Krippahl (1960), and has been well documented by Izawa (1962), Good (1963), Vennesland *et al.* (1965), West and Hill (1967) and Stemler and Govindjee (1973), among others. It now appears that bicarbonate ion (HCO_3^-), not dissolved CO_2 gas, is the active form in this phenomenon (Stemler and Govindjee, 1973). Despite its ability to stimulate the Hill reaction, HCO_3^- was thought not to be directly involved in the primary photochemical reactions associated with oxygen evolution, as the HCO_3^- effect was not always observed at low light intensities (Izawa, 1962; Good, 1963). Since these observations could also be explained by assuming incomplete removal of HCO_3^-, the role of this ion in photosynthetic oxygen evolution remained ambiguous. Recently, Stemler and Govindjee (1973) found that at least one site of action of HCO_3^- occurs between the oxygen-evolving site and the electron-donation site of the artificial donor diphenyl carbazide (DPC) to photosystem II. This new evidence indicates HCO_3^- may be involved in the initial photochemical reactions leading to oxygen evolution. Further study, using maize chloroplasts, was therefore undertaken to determine the effects of HCO_3^- on chlorophyll *a* (Chl *a*)

fluorescence transients and delayed light emission (DLE), both reflecting initial photochemical events in photosystem II.

MATERIALS AND METHODS

Maize (*Zea mays*) chloroplasts were isolated and depleted of bound bicarbonate in a manner already described by Stemler and Govindjee (1973). The suspension medium is described in the legends of Figs. 1, 5, and 7.

Fluorescence-transient measurements were made as described by Munday and Govindjee (1969). The photomultiplier signal was fed through a Tektronix oscilloscope (type 502) and displayed on an Esterline Angus (model E 11015) or a Midwestern oscillograph recorder (model 801). Chlorophyll *a* fluorescence was excited with blue-green light (Corning C.S. 4–96 plus 3–73 filters). The light intensity was 80 W/m² or lower, reduced by means of calibrated neutral-density filters. Fluorescence was observed at 685 nm (half band width, 6·6 nm) through a Bausch & Lomb monochromator (blazed at 750 nm). A Corning C.S. 2–58 filter, placed at the entrance slit of the analyzing monochromator, eliminated stray exciting light. Recovery of the fluorescence transient in the dark was measured as the area bounded by the fluorescence transient curve after variable periods of darkness, divided by the area after a long dark period, times 100 (see Mohanty *et al.*, 1971).

Measurements of DLE were made using the instrument described by Jursinic and Govindjee (1972). For these measurements, chloroplast suspensions were pre-illuminated with blue light (Corning C.S. 4–96 filter). The intensity of exciting light was 0·4 W/m² or 8·0 W/m² (in some experiments). Both DLE and fluorescence measurements were taken under anaerobic conditions.

RESULTS AND DISCUSSION

Chlorophyll a fluorescence transients

High light intensity. Figure 1 shows the fast fluorescence transient observed in isolated chloroplasts exposed to 80 W/m² blue-green light. These traces were obtained with an Esterline Angus recorder. Careful and repeated measurements, using the faster oscillographic recorder, showed no effect of bicarbonate ions on the "O" level (i.e., the constant fluorescence; Fo). However, in bicarbonate-depleted chloroplasts, the initial fluorescence rise from the first recorded level to I (intermediate hump) fluorescence is rapid, but the I → D (the inflection) and the D → P (peak, F∞) phases are slow. In the presence of 10 mM bicarbonate, the initial fast rise to I appears slower and is depressed, the level D is not clear and occurs earlier (see insert Fig. 1), and the D → P rise is much more rapid with a half rise time of about 1 s vs 4 s in bicarbonate-depleted chloroplasts. The intensity of fluorescence at the P level, like that at the O level, is insensitive to HCO_3^-. In saturating light (as used here), when 3-(3,4-dichlorophenyl)-1,1 dimethylurea (DCMU) is present, the fluorescence transient is not noticeably different with and without HCO_3^-. The rise to P is very rapid, as expected, compared to the unpoisoned chloroplasts, but the extent of the P level is lower by about 25 per cent in both cases.

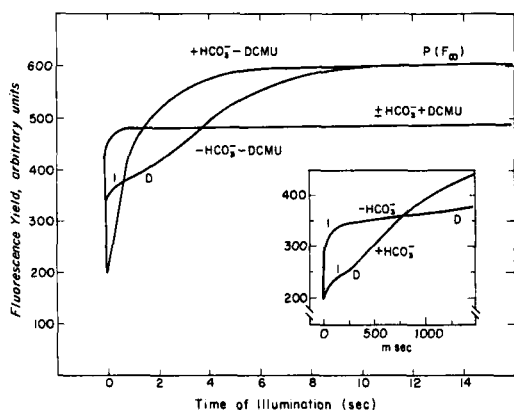To explain Chl *a* fluorescence transients, it is generally assumed that the yield of variable fluorescence is a function of the redox state of the primary electron acceptor of photosystem II, Q (Duysens and Sweers, 1963). HCO_3^- depletion may pose a block in electron flow either before or after Q. If the block occurs after Q, however, thus preventing the reoxidation of Q by intersystem intermediates (A pool), one would expect variable fluorescence to be at all times greater in the absence of HCO_3^-. This is not the case. The I to P phase is instead retarded by HCO_3^- depletion. Thus it is suggested that HCO_3^- acts prior to Q on the oxygen-evolving side of photosystem II, in agreement with our earlier conclusion (Stemler and Govindjee, 1973). As a working hypothesis, we suggest that HCO_3^- may be affecting the ratio of the oxidation states S_0/S_1 (referring to the kinetic model and notation of Forbush *et al.*, 1971). This ratio can be changed chemically (Bouges-Bocquet, 1973). More specifically, the S_0/S_1 ratio may be higher in the absence of HCO_3^- than in its presence, HCO_3^- acting to stabilize the S_1 state. The only additional assumption required is the existence of an endogenous electron donor D_0^- (already proposed by Forbush *et al.*, 1971 to explain dark deactivation of oxidation states S_2 and S_3). In the absence of HCO_3^-, the faster OI rise is explained to be due to the $S_0 \rightarrow S_1$ transition and the slower IP rise to the slow donation of electrons by D_0^- to the S_1 state, reforming the S_0, eventually reducing the A pool completely. In the presence of HCO_3^-, the relatively smooth OP rise reflects the normal transitions from the S_1 state (stabilized by HCO_3^-) to all higher states until the A pool is likewise completely reduced. (For a detailed model, see Stemler, Ph.D. thesis, in preparation.)

If unpoisoned chloroplasts are subjected to 80 W/m² light until the P level is achieved, recovery of the fluorescence transient is incomplete, regardless of the dark time allowed (up to 10 min; see Fig. 2). This may be due to the anaerobic conditions used, which would prohibit the reoxidation of electrons acceptors by oxygen or to a non-specific effect of the high salt concentrations employed. Recovery is, however, faster and more complete in the presence of HCO_3^-. After one min of darkness, about 8·5 per cent of the transient is recovered in HCO_3^--depleted chloroplasts, while about 17 per cent is recovered in chloroplasts given 10 mM HCO_3^-; after 10 min dark, values are 35 and 60 per cent, respectively.

Low light intensity. Fluorescence transients were also measured at low light intensity (0·4 W/m²) in order to allow comparison with DLE results to be reported here. At this intensity, fluorescence is much greater in the presence of DCMU (see Fig.

Figure 1. Fluorescence yield of chlorophyll *a* at 685 nm as a function of time of high-intensity illumination in the presence and absence of 0·01 *M* NaHCO₃. Maize chloroplasts previously depleted of HCO_3^- were suspended in 0·25 *M* NaCl, 0·04 *M* Na acetate, 0·05 *M* phosphate buffer pH 6·8, ±10⁻⁵ *M* DCMU; 10 μg chlorophyll/ml. Blue actinic light, 80 W/m².
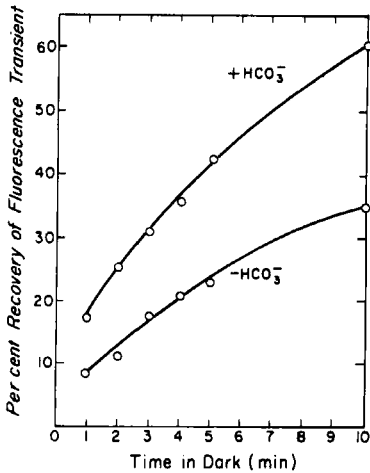
Figure 2. Recovery of the chlorophyll *a* fluorescence transient in the dark using unpoisoned chloroplasts. Conditions were the same as in Fig. 1.

3). HCO_3^- slows the half rise time to steady state by about 30 per cent, and lessens the extent of rise by about 15 per cent. In the absence of DCMU, very little variable fluorescence is observed although a slightly greater amount is seen in the absence of HCO_3^-.

An effect of HCO_3^- at this low light intensity and in the presence of DCMU stresses once again that HCO_3^- is affecting early photochemical events. The lower steady-state (P) level of fluorescence seen in the presence of both HCO_3^- and DCMU may be explained by proposing a rapid back reaction (charge recombination) in the reaction centers exposed to HCO_3^-. Thus reaction centers would be 'reopened' at an appreciable rate compared to

their rate of closing by light, suppressing the variable fluorescence. This idea is supported by both a faster rate of recovery of the fluorescence transient and a much larger amount of delayed light emission observed in the presence of both HCO_3^- and DCMU (*vide infra*).

The recovery of low-intensity fluorescence transients was measured, using DCMU-poisoned chloroplasts, with dark times up to 16 s only (Fig. 4). In the presence of HCO_3^-, about 14 per cent of the transient is recovered within 2 s. Very little additional recovery is seen in the following 14 s. In the absence of HCO_3^-, only 2–3 per cent of the transient is recovered within 2 s. Recovery increases to about 8 per cent at 6 s and increases slowly to 10 per cent at 16 s. Even after long periods of dark, recovery of the transient remains far from complete, whether or not HCO_3^- is present. After 2 min (data not shown), only about 35 per cent of the transient is recovered, and this is the same in the presence and absence of HCO_3^-. It must be pointed out that a lower steady-state (P) level was used in calculating recovery (for Fig. 4) in the presence of bicarbonate than in its absence, thus reducing the area over the curve. This is significant because we propose that the lower P level in the presence of HCO_3^- is due to a rapid primary back reaction, i.e., charge recombination, taking place. Consequently, more photosystem II traps stay open during illumination in the presence of HCO_3^-, suppressing variable fluorescence. Since recovery was calculated on the basis of what occurred in the dark, only the per cent of photo-
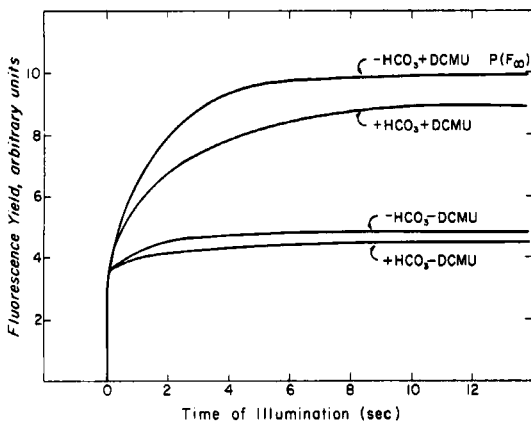

Figure 3. Fluorescence yield of chlorophyll *a* at 685 nm as a function of time of low-intensity illumination. Blue actinic light, $0.4 \, W/m^2$. Other conditions were the same as in Fig. 1.
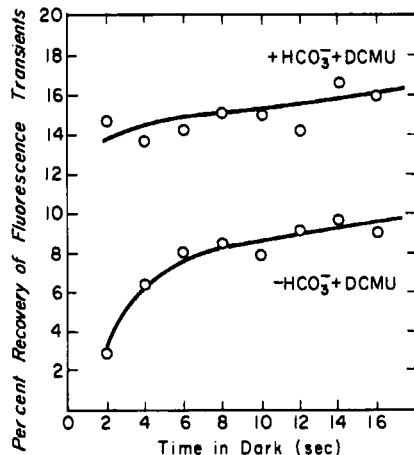

Figure 4. Recovery of the chlorophyll *a* fluorescence transient in the dark using DCMU poisoned chloroplasts after illumination with weak blue light, $0.4 \, W/m^2$. Other conditions were the same as in Fig. 1.

synthetic units actually closed at the termination of illumination are considered in the recovery data. In other words, the per cent of traps capable of very fast recovery in the presence of HCO_3^- is probably much higher than the 14 per cent indicated in Fig. 4. In any case, we can make the qualitative conclusion that back reactions of the PS II centers are facilitated by HCO_3^-.

Delayed light emission

Figure 5 shows the delayed light emitted, by chloroplasts, seconds after illumination for 10 s with 0·4 W/m² blue light. The greatest amount of delayed light is observed when chloroplasts are illuminated in the presence of both HCO_3^- and DCMU. When DCMU is absent, the intensity of DLE is reduced by slightly more than 50 per cent. Still less (about 25 per cent of the maximum) is observed when both HCO_3^- and DCMU are omitted. However, the least DLE (about 10 per cent of the maximum) is seen when HCO_3^- is omitted but DCMU is present.

When chloroplasts are illuminated for 60 s at low intensity (0·4 W/m²), the pattern of DLE is different (Fig. 6). The amount of DLE is greatest in the presence of HCO_3^- but in the absence of DCMU. A large increase (about 3-fold) is seen compared to the 10 s value. Relatively less (about 70 per cent of the maximum) DLE is observed
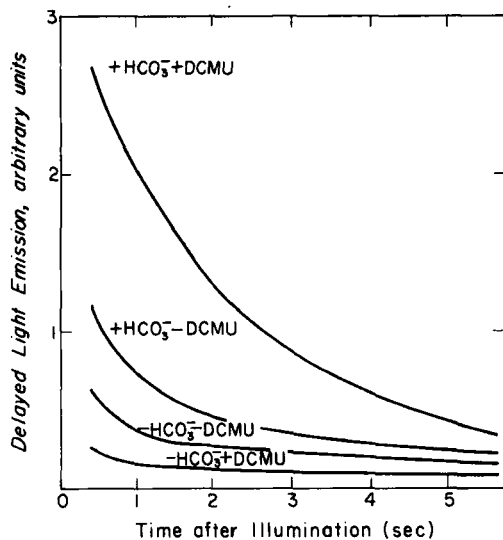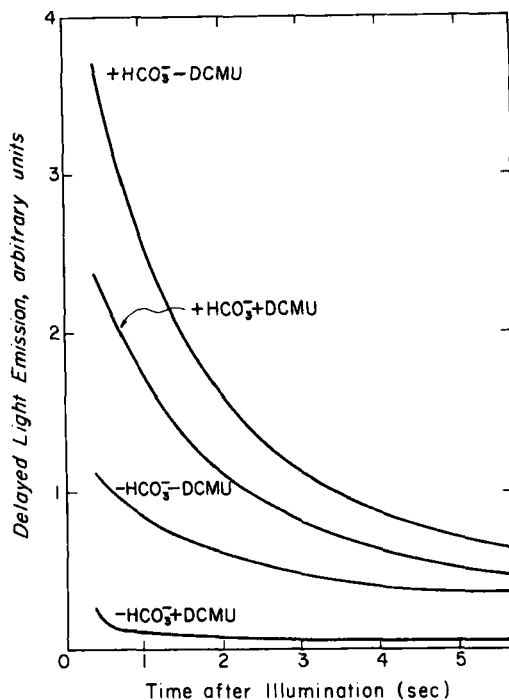

Figure 6. DLE decay from maize chloroplasts illuminated for 60 s. All other conditions exactly as described in the legend of Fig. 5.

Figure 5. DLE (delayed light emission) decay from maize chloroplasts, previously depleted of HCO_3^-, illuminated for 10 s in weak blue light (0·4 W/m²). Chloroplasts were suspended in 0·25 M NaCl, 0·04 M Na acetate, 0·05 M phosphate buffer pH 6·8, ±0·01 M NaHCO₃ ± 10⁻⁵ M DCMU. 15 μg chlorophyll/ml.

when HCO_3^- and DCMU were both present, as this amount of DLE remained essentially unchanged from the 10 s value. The amount of DLE seen in the absence of both HCO_3^- and DCMU increased (about 2-fold) from the 10 s value but remained less (about half as much) than observed in the presence of both treatments. The least amount of DLE (about 5 per cent of maximum) is observed in the absence of HCO_3^- but in the presence of DCMU; here an actual decline from the 10 s value occurs.

Short periods (10 s) of higher illumination (80 W/m², data not shown) produced results very similar to longer periods (60 s) of weak light (Fig. 6).

While it is obvious that HCO_3^- is stimulating back reactions in the PS II reaction centers, the data present interesting complications. DLE is extremely low by about 10 s after termination of the exciting beam, yet recovery of the fluorescence transient, in DCMU-poisoned systems, is far from complete at this time in our samples. Presumably, a large population of reduced Q still exists when the back reactions leading to DLE have almost ended. Perhaps an endogenous electron donor on the oxygen-evolving side of photo-

system II is competing with Q^- for Z^+ quite successfully and acting in the same manner as artificial electron donors such as hydroxylamine in quenching DLE (Bennoun 1970; Stacy *et al.*, 1971, Mohanty *et al.*, 1971).

Delayed light emission induced by DCMU injection

If DCMU is injected into a chloroplast suspension as DLE is being monitored after a 10 s illumination with weak blue light, a large increase in light emission results (Fig. 7). This effect was first observed by Clayton (1969) and confirmed by Lavorel (1973). We see, however, that HCO_3^- must be present during illumination to observe this effect. Our new finding is that bicarbonate-depleted chloroplasts show no DLE jump upon injection of DCMU. After 60 s of the same illumination, a slightly different effect is seen (Fig. 8). Chloroplasts illuminated in the presence of HCO_3^- show a less dramatic DLE jump, and HCO_3^--depleted chloroplasts demonstrate an actual drop in DLE upon injection with DCMU.

The opposite experiment, injecting HCO_3^- while monitoring DLE from HCO_3^--depleted chloroplasts, was also done. No effect on DLE was observed upon injection of HCO_3^- whether or not the chloroplasts were poisoned with DCMU during illumination (data not shown). Short (10 s) periods of illumination subsequent to HCO_3^- injection did yield an increased DLE, however. It appears, therefore, that HCO_3^-, unlike DCMU, must be present during illumination to effect DLE.
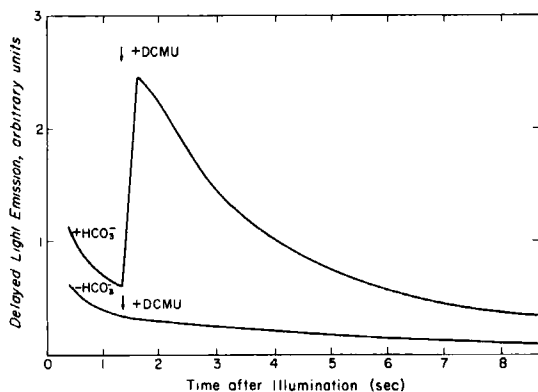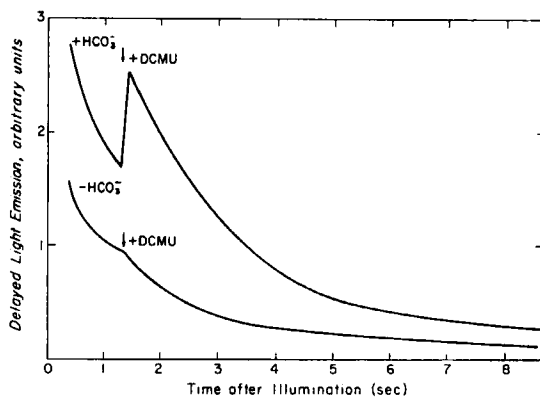

Figure 8. DLE decay from maize chloroplasts illuminated for 60 s. All other conditions exactly as described in the legend of Fig. 7.

Clayton (1969) explained the gush of DLE seen upon injection of DCMU as simply the result of a jump in the quantum yield of fluorescence, implying that there was no increase in the rate of charge recombination *per se*. However this idea does not explain why there is a lack of DLE gush when DCMU is added in the absence of HCO_3^-. To explain this phenomenon, we may postulate that in the absence of HCO_3^-, the redox state of Z may be inappropriate for the primary back reaction to occur.

CONCLUDING REMARKS

The effects of HCO_3^- on initial photochemical events seem in some ways contradictory. While HCO_3^- is known to stimulate oxygen evolution (Good, 1963) and ferricyanide and DCPIP reduction (West and Hill, 1961; Stemler and Govindjee, 1973), indicating greater efficiency of PS II, back reactions leading to recovery of Chl *a* fluorescence transients and DLE are also stimulated, arguing for less over-all efficiency. To render these observations compatible, we propose that HCO_3^- permits the formation of higher oxidation states ($> S_1$) of the PS II reaction centers. Such states are not only necessary for oxygen evolution, according to the kinetic model of Forbush *et al.* (1971) but are also the only states with a significant propensity to decay (back react) yielding much more DLE (Joliot *et al.*, 1971; Barbieri *et al.*, 1970). Specifically we suggest, as a working hypothesis, that HCO_3^- may do this by somehow stabilizing the S_1 state.

Whatever may be the precise role of HCO_3^- in oxygen evolution, the fact that it markedly affects the fast Chl *a* fluorescence transient, de-

Figure 7. DLE decay from maize chloroplasts previously depleted of HCO_3^-, illuminated for 10 s in weak blue light (0·4 W/m²), 0·2 ml DCMU injected where indicated. Final concentrations, 0·25 *M* NaCl, 0·04 *M* Na acetate, 0·05 *M* phosphate buffer pH 6·8, ±0·01 *M* NaHCO₃, 15 μg chlorophyll ml⁻¹.

layed light emission, reactions between the oxygen-evolving site and reaction center II (Stemler and Govindjee, 1973), in addition to its possible role in photophosphorylation (Punnett and Iyer, 1964; Batra and Jagendorf, 1965), and that it even does so at low light intensities, allows the assertion that HCO_3^- is of critical importance in the initial photochemical processes of photosynthesis.

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