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A SECONDS RANGE COMPONENT OF THE REOXIDATION OF THE PRIMARY PHOTOSYSTEM II ACCEPTOR, Q

EFFECTS OF BICARBONATE DEPLETION IN CHLOROPLASTS *

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Broken chloroplasts depleted of bicarbonate (HCO_3^-) show 30–50% inhibition of the Hill reaction in low-intensity light. Also, photoreactions excited by repetitive flashes measured by oxygen evolution, ESR signal IIvf, and absorption changes at 680 and 334 nm show inhibition of 30–50%. An effect of HCO_3^- was sought to explain these phenomena. The decay of chlorophyll *a* fluorescence yield in the millisecond and seconds range, following a single flash, was observed to be multiphasic with a very slow component of 1–2 s half-time. In HCO_3^--depleted samples this component is enhanced 2- or 3-fold. Since this occurs even after one flash, it is suggested that HCO_3^- affects the $Q^-B \rightarrow QB^-$ reaction. In this work it is shown that 40% inhibition of oxygen flash yield is relieved to a great extent if the excitation flash rate is decreased from 2 to 0.33 Hz. A measurement of 520 nm absorption change in the presence of ferricyanide, which is proportional to Photosystem II charge separation, shows a similar inhibition that is dependent on flash rate. The maximum amplitude of variable fluorescence yield and 520 nm absorption change after a single flash are unaffected by HCO_3^- depletion. The dark distribution of oxygen-evolution S-states is found to be shifted to a more reduced configuration in depleted samples. It is concluded that normal charge separation occurs in HCO_3^--depleted Photosystem II reaction centers but that a large fraction of Q^- decays so slowly that not all Q^- is reoxidized between flashes given at a rate of 1 or 2 Hz. Thus, a portion of the Photosystem II centers would be closed to photochemistry. There is a reversible effect of HCO_3^- depletion on the oxygen-evolution system that is observed as a shift in the dark distribution of S-states.

Introduction

It has been established that HCO_3^- in catalytic amounts can modify PS II activity. Detailed reviews of various aspects of this subject may be found elsewhere [1–3]. In chloroplasts that have been depleted of HCO_3^-, electron flow as measured by the Hill reaction is inhibited by 80–90% in saturating light [4–9]. The extent of the inhibition is dependent upon excitation-light intensity and is 30–50% under weak light [8]. Also, Stemler et al. [8] reported that chloroplasts depleted of HCO_3^- had the S-state transitions of their oxygen-evolution system slowed to 4 ms half-time. It was originally felt that the site of HCO_3^- action was primarily on the oxygen-evolution side of PS II [5]. More recent work [9–14] has established

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[*] The mention of firm names or trade products does not imply that they are endorsed by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; Chl, chlorophyll.

that the major site of HCO_3^- action is on the reducing side of PS II. The slowdown of the S-state transitions was explained by the hypothesis of Jursinic et al. [10], which suggested that HCO_3^- depletion slowed the $Q^-B \rightarrow QB^-$ reaction to 3 ms half-time. Q and B represent the primary and secondary electron acceptors of PS II, respectively. The observed slow rate of the $Q^-B \rightarrow QB^-$ reaction in depleted samples could not explain the 80–90% inhibition in the Hill reaction, since in normal chloroplasts the rate-limiting step in electron flow has a 20 ms half-time [15]. This was explained by another effect of depletion; the slowdown of the $Q^-B^{2-}A \rightarrow QB^-A^{2-}$ reaction to a 150 ms half-time [11], where A represents the plastoquinone pool. However, another effect of HCO_3^- depletion could not be explained by the above hypotheses. This was a 30–50% inhibition in charge separation indicated by a variety of repetitive flash-excitation measurements [8,10,12], and the 30–50% inhibition of the Hill reaction under low-intensity light [8]. An inactivation of PS II reaction centers was suggested [10] as an explanation for these effects.

In this work, observations of a fluorescence decay component with a half-time of seconds are reported. This component is enhanced 2- to 3-fold in depleted samples even after one excitation flash. This indicates that the $Q^-B \rightarrow QB^-$ reaction is involved. The occurrence of a large slow component of Q^- reoxidation in depleted chloroplasts provides an explanation for the 30%–50% inhibition observed in repetitive flash-excitation measurements and oxygen evolution under low-intensity illumination. Such decreases can now be explained by accepting that a portion of the reaction centers remains in a photochemically closed state due to slow Q^- reoxidation. The cause of this enhancement in the slow Q^- reoxidation is believed to be an altered equilibrium between Q and B. The reason for such an equilibrium change, and why only a portion of the reaction centers shows the very slow Q^- reoxidation, is not clear.

Methods

Chloroplast preparation

Broken chloroplasts were isolated from leaves of dwarf peas (*Pisum sativum*) grown in the laboratory and harvested 14–21 days after germination. The isolation procedure was as previously described [16]. Both freshly prepared and frozen samples that were kept under liquid nitrogen were used. Unless otherwise stated, the chloroplasts were depleted of HCO_3^- by the low-pH method [17], and the reaction mixture consisted of 50 mM phosphate (pH 6.8), 200 mM NaCl and 1 mM ferricyanide. It is important to note that this reaction medium does not contain formate. See Results for a discussion of this point. All measurements were carried out at a temperature of 20°C.

Oxygen flash yield

A rate electrode was used for oxygen flash-yield measurements as previously described [16]. Xenon flash excitation was given at a 2 Hz rate unless stated otherwise. The calculation method used for determining the S-state dark distribution and transition parameters was as described by Jursinic [18].

Fluorescence yield

In the range of 50 μs to 500 ms the fluorescence yield was measured by the two-flash method [10]. The actinic flash was provided by a General Radio Strobotac 1538-A source through a Corning CS 4-96 glass filter. The delayed analytic flash was identical to the actinic flash, except that it was reduced in intensity by a 1% neutral density filter. For the 500 ms to 40 s range the analytic flash was replaced by a low-intensity tungsten lamp, whose light was passed through a Corning 4-96 glass filter and an electronic shutter with 0.8 ms opening time. The intensity of this continuous analytic light was low enough that it caused no measurable amount of Q reduction during the period of measurement. Fluorescence was detected by a Hamamatsu R928 photomultiplier that was shielded from actinic light with a Corning CS 2-64 glass filter. The analog photomultiplier output was digitized by a Biomation 805 waveform recorder. For measurements made with the analytic flash, digital data were transferred to a Heath H8 minicomputer, which had been programmed to determine peak heights. For measurements made with continuous analytic light, the data were displayed on a chart recorder.

520 nm absorption change

Absorption changes at 520 nm were measured with a conventional single-beam spectrophotometer, which has a 200 μs electronic rise time. Single saturation flashes were provided by two xenon flashes, one on each side of the sample cuvette, that were triggered simultaneously. The analytic light was from a tungsten lamp and was passed through a 520 nm interference filter and electronic shutter. The shutter was opened 60 ms prior to the actinic flash in order to avoid large amounts of excitation by the analytic light. The photomultiplier was protected from actinic light by a 520 nm interference filter. Data were digitized as in the fluorescence measurement, and improvement in signal-to-noise ratio was accomplished by averaging a number of data traces with the minicomputer.

Delayed light emission

Delayed light emission was measured beginning at 100 μs, following flash illumination. The saturating flashes of 400 ns pulse width were provided by a Phase-R model 1100 dye laser. The laser was operated with Phase-R LD490 dye in ethanol, which has a peak emission wavelength of 479 nm. The photomultiplier was gated off electronically by a factor of $5 \cdot 10^3$ during the actinic flash. Further protection of the photomultiplier from actinic light was provided by a Corning 2-64 glass filter. Artifactual signals from scattered light and from sample fluorescence were demonstrated to be less than 1% of the delayed light emission signal amplitude. Delayed light emission was measured in four ranges of 100 μs to 1 ms, 1 ms to 10 ms, 10 ms to 100 ms, and 100 ms to 4 s. Electronic rise times of 3 μs, 30 μs, 300 μs and 2.2 ms were used in each measuring range, respectively. Data were digitized as in the fluorescence measurement and converted to numerical form by the minicomputer.

Results

Fluorescence-yield changes

The extent of variable fluorescence yield generated by a single saturating excitation flash is proportional to the number of active PS II reaction centers if energy transfer does not occur between closed and opened reaction centers. This was demonstrated to be the case in our HCO_3^--depleted and reconstituted preparations. We observe that the rise in fluorescence yield upon continuous illumination with DCMU present is exponential and not sigmoidal. This result is in agreement with earlier fluorescence-transient results of Stemler and Govindjee [7]. When the variable fluorescence yield is measured at 50 μs after a flash, appreciable Q^- reoxidation has not yet occurred and any artifacts caused by carotenoid triplets have decayed away [19,20]. For HCO_3^--depleted samples $\Delta F = [F(50\ \mu\text{s})/F_0] - 1 = 2.1$, where ΔF is the variable fluorescence yield, $F(50\ \mu\text{s})$ the fluorescence signal amplitude at 50 μs after the flash, and F_0 the fluorescence level in a dark-adapted sample prior to excitation. For samples with 10 mM HCO_3^- added, $\Delta F = 2.0$. This almost identical amount of variable fluorescence indicates that no appreciable decrease in the number of active PS II reaction centers occurs due to HCO_3^- depletion. The fluorescence decay in the 50 μs to 1 ms range for samples with or without HCO_3^- was found to be identical to what has been reported previously [10].

The decay of fluorescence in the 1 ms and

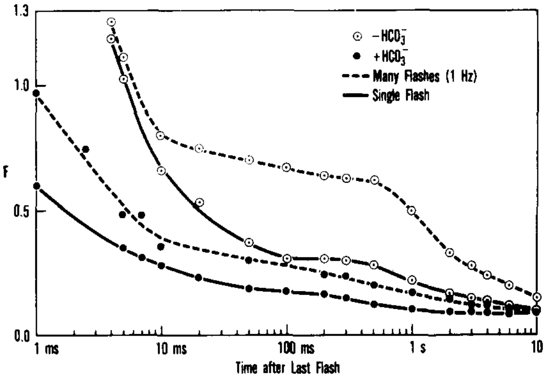

Fig. 1. The decay of variable fluorescence, ΔF, vs. the time after a saturating excitation flash. The variable fluorescence is calculated by the following formula: $\Delta F = [F(t)/F_0] - 1$ where $F(t)$ is the fluorescence amplitude at time t after the excitation flash and F_0 is the fluorescence amplitude in a dark-adapted sample. Fluorescence decays are shown for the following samples: HCO_3^- depleted after a single flash (○——○) and after many flashes (○------○), and +10 mM HCO_3^- after a single flash (●——●) and after many flashes (●------●). The chloroplast concentration for these measurements was 10 μg Chl/ml. The flash rate was 1 Hz.

greater range in depleted samples has not been reported previously. Fluorescence decay in this time range after a single flash is shown in Fig. 1. Even after one flash the depleted sample has a much greater ΔF in all time ranges due to slower decay kinetics. In the 50 μs to 10 ms range the half-time of decay is approx. 6 ms, and in the 10 ms to 5 s range it is approx. 1–2 s for the depleted sample. Much slower components also exist, and the variable yield returns to zero in about 4–5 min. Fig. 2A and B shows that with flashes given at a rate of 1 Hz, the fluorescence-yield amplitude increases gradually with flash number in samples with HCO_3^- present and more rapidly in samples depleted of HCO_3^-. This fluorescence increase is distinct from that reported by Govindjee et al. [11]. The larger variable yield reported here in depleted samples occurs even on the first flash and on subsequent flashes given at a rate of 1 Hz. The increased variable yield previously reported [11] was seen only after the third flash and if flashes were given at a rate of 33 Hz. An increase in variable yield of this type could be observed in the samples used in this work if flashes were given at 10 Hz (see Fig. 2A). Thus, there is an extra variable yield that occurs at high flash rates and can be observed after the third flash. However, it is in addition to a variable yield that already is present even after one flash and can be observed when flashes are given at a rate of 1 Hz.

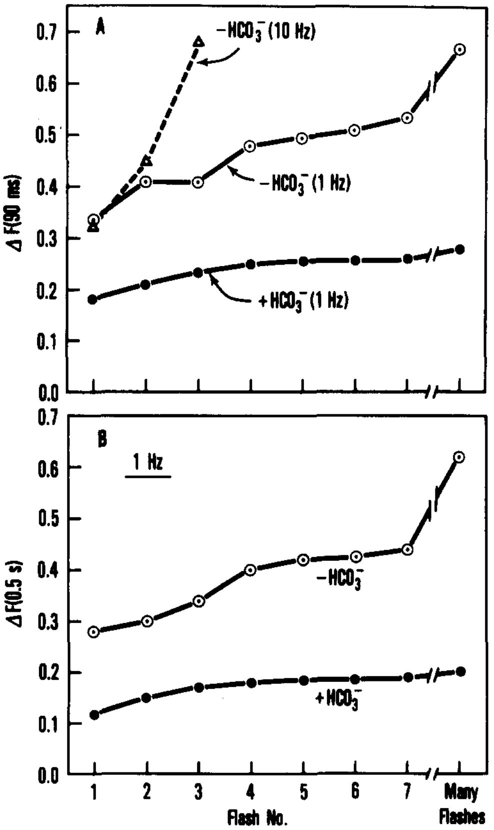

Fig. 2. The amplitude of the variable fluorescence yield, ΔF, vs. flash number. The variable yield was calculated as described in the legend to Fig. 1. Excitation flashes were given at a rate of 1 Hz except for data points indicated by (△), for which a 10 Hz rate was used. (A) ΔF at 90 ms after the excitation flash for HCO_3^--depleted samples (○———○) and (△———△) and + 10 mM HCO_3^- samples (●———●). (B) ΔF at 0.5 s after the excitation flash for HCO_3^--depleted samples (○———○) and + 10 mM HCO_3^- samples (●———●). The chloroplast concentration for these measurements was 10 μg Chl/ml.

The variable yield of fluorescence has been shown [21] to arise from PS II reaction centers with their primary acceptor Q in its reduced state, Q^-. Centers in the P-680-Q^- state are closed to photochemistry. Thus, depleted samples will have a portion of their reaction centers in a closed state for many seconds after an excitation flash. Any experiment that depends on repeated flashes for excitation will be expected to show an inhibition in depleted samples that will be dependent upon flash rate. Table I indicates values for f, the portion of total variable fluorescence remaining at 0.5, 1.0 and 3.0 s after the last flash of a sequence of 25 flashes. These values of time correspond to the flash periods for flashes given at 2, 1 and 0.33 Hz, respectively. Values for $1-f$ are also shown and, according to the principle of the fundamental alternative [22], they are indicative of the portion of maximal amount of the photochemical reaction that can take place in samples that do not have energy transfer between closed and open centers. The values shown in this table indicate that depleted samples receiving flashes at a rate of 2, 1 and 0.33 Hz will have 30, 24 and 13% of their reaction centers not recovering between flashes, respectively. Therefore, one might expect the extent of inhibition by HCO_3^- depletion to be near these amounts at these flash rates.

Oxygen flash yield

It has been previously shown [8] that the oxygen

TABLE I

PORTION OF TOTAL VARIABLE FLUORESCENCE AT VARIOUS TIMES AFTER THE EXCITATION FLASH

The portion of total variable fluorescence, f, is calculated by the following formula:

$$f=\frac{F(t)-F_0}{F_{\max}-F_0}$$

where $F(t)$ is the maximum fluorescence amplitude and is measured at 50 μs after the excitation flash, and F_0 is the fluorescence amplitude in a dark-adapted sample. The values calculated are for chloroplasts that have been illuminated by 25 flashes. R is the ratio of $1-f$ values for +10 mM HCO_3^- to HCO_3^--depleted samples.

Time after the excitation flash (s)	$-HCO_3^-$		$+HCO_3^-$		R
	f	$1-f$	f	$1-f$	
0.5	0.30	0.70	0.10	0.90	1.29
1.0	0.24	0.76	0.08	0.92	1.21
3.0	0.13	0.87	0.06	0.94	1.08

flash yield at steady state was inhibited by approx. 40% in depleted samples that were given flashes at a 1 Hz rate. This type of experiment is repeated here but with flash rates of 2, 1 and 0.33 Hz, and typical oxygen-yield patterns are shown in Fig. 3. Mathematical analysis of oxygen flash-yield patterns in five separate measurements for each flash rate gave average parameters shown in Table II.

The differences in the amplitude of the oxygen flash yield between ± HCO_3^- samples are observed to become smaller as the flash rate is decreased. This is most clearly seen in the ratio of steady-state yields shown in Table II. The relationship between $1-f$ ratio of Table I and the Y_{ss} ratios of Table II is quite striking. Another more subtle change in the oxygen flash-yield pattern due to HCO_3^- depletion is a shift in the dark S-state distribution. In HCO_3^--depleted samples the $S_0^{(0)}$ state is 0.4 (see Table II). Adding HCO_3^- in the dark restores a normal S-state distribution, and the $S_0^{(0)}$ state becomes 0.25. This difference is significant and occurs even in the first flash sequence observed 5 min after HCO_3^- has been added in the dark.

It should be noted that the inhibition in steady-state oxygen for depleted samples given flashes at 2 Hz does not correspond with a signifi-

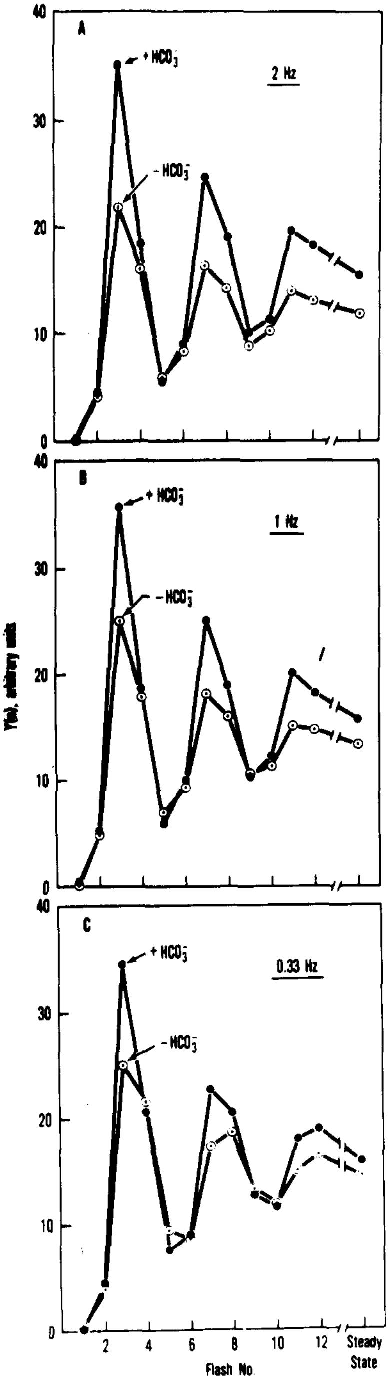

Fig. 3. Oxygen flash yield, $Y(n)$, as a function of flash number following 5 min of dark adaptation. The $Y(n)$ values, while given in arbitrary units, are all on the same relative scale. Chloroplasts were depleted of HCO_3^- (○——○) or had 10 mM HCO_3^- added (●——●). Excitation flashes were given at a rate of 2 Hz (A), 1 Hz (B), and 0.33 Hz (C). Steady state was attained after approx. 20 flashes. Chloroplasts were placed on the electrode at a concentration of 0.5 mg Chl/ml.

TABLE II

OXYGEN-EVOLUTION TRANSITION PARAMETERS FOR $\pm HCO_3^-$ CHLOROPLASTS WITH VARIOUS EXCITATION FLASH RATES

Homogeneous transition parameters for oxygen-evolution S-states, calculated as described in the text from oxygen flash-yield patterns: α is the miss, β is the single-hit, and γ is the double-hit parameter. The corresponding dark adapted S-state ($S_0^{(0)}$, 1, 2) distribution is also shown. In all cases, $S_2^{(0)}$ was found to equal zero. These values are averages of at least five separate determinations of the flash-yield patterns as in Fig. 3A–C, and standard deviations are indicated. R is the ratio of the Y_{ss} values for the +10 mM HCO_3^- to HCO_3^--depleted samples.

Sample conditions	α	β	γ	$S_0^{(0)}$	$S_1^{(0)}$	Y_{ss}	R
$-HCO_3$, 2 Hz	0.086±0.009	0.833±0.020	0.081±0.010	0.42±0.03	0.58±0.03	11.9±0.40	–
$-HCO_3^-$, 1 Hz	0.101±0.011	0.822±0.021	0.077±0.010	0.38±0.03	0.62±0.02	13.4±0.50	–
$-HCO_3^-$, 0.33 Hz	0.129±0.011	0.811±0.020	0.060±0.009	0.42±0.03	0.58±0.03	14.6±0.40	–
+10 mM HCO_3^-, 2 Hz	0.087±0.009	0.862±0.018	0.051±0.006	0.25±0.02	0.75±0.02	15.4±0.40	1.29
+10 mM HCO_3^-, 1 Hz	0.086±0.007	0.857±0.020	0.057±0.007	0.25±0.02	0.75±0.02	15.8±0.30	1.18
+10 mM HCO_3^-, 0.33 Hz	0.116±0.010	0.835±0.009	0.049±0.009	0.25±0.02	0.75±0.02	16.0±0.40	1.10

cant increase in the miss parameter. This is puzzling and may mean that only a fraction of the centers are responsible for the slow phase in the fluorescence decay.

520 nm absorption change

The absorption change at 520 nm is due to an optical band-shift of membrane pigments in an electric field [23,24]. The electrical field across the membrane and the associated absorption change are proportional to the amount of charge separation that occurs in PS I and PS II [25]. In order to use the 520 nm absorption change is an indication of the amount of PS II charge separation, PS I photoreaction can be eliminated chemically by inclusion of DCIP and ferricyanide [25], which maintains the plastoquinone pool and P-700 in oxidized form.

The 520 nm absorption change due to PS II charge separation in control chloroplasts and $\pm HCO_3^-$ chloroplasts is shown in Fig. 4. To do these experiments, it was not possible to use the usual depletion procedure (see Methods), as this was found to virtually eliminate the 520 nm absorption change. This corroborates an earlier report [13] that washing cloroplasts in pH 5 buffer to deplete them of HCO_3^- also causes uncoupling. Instead of the pH 5 treatment, the chloroplasts were illuminated in the presence of formate at pH 6.8 for a short time. This also induces HCO_3^- dependence [26], but does not uncouple the chlo-

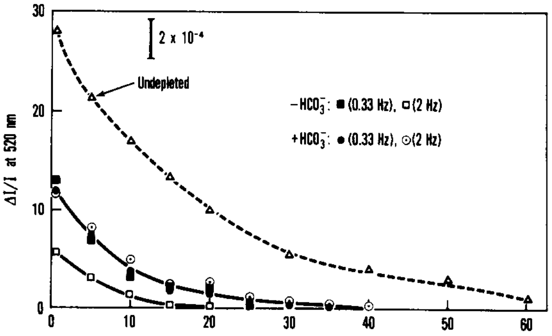

Fig. 4. Absorption change at 520 nm vs. time after the excitation flash. Decays are shown for chloroplasts that are undepleted (△ - - - △), HCO_3^- depleted given flashes at a rate of 0.33 Hz (■——■) and 2 Hz (□——□), and +10 mM HCO_3^- given flashes at a rate of 0.33 Hz (●——●) and 2 Hz (○——○). The chloroplasts were depleted of HCO_3^- by the pH 6.8 with illumination method [26]. The reaction medium consisted of 50 mM phosphate (pH 6.8), 100 mM NaCl, 100 mM sodium formate, 3 mM ferricyanide and 50 μm DCIP. Measurements were made at a chloroplast concentration of 50 μg Chl/ml. Averaging of 16 decays was carried out to improve the signal-to-noise ratio.

roplasts to as great an extent. Some uncoupling was observed, however, as evidenced by the lower amplitude of the 520 nm absorption change in HCO_3^- reconstituted versus undepleted samples in Fig. 4. This uncoupling by HCO_3^- depletion is unusual in that some of the thylakoids will have membrane potentials with lifetimes less than 200 μs (the machine rise time) while others will have the longer lifetimes shown in Fig. 4. Another possibility is that depletion causes some thylakoids to have charge separation that is nonelectrogenic.

In depleted chloroplasts, the amplitude of the 520 nm absorption change is affected by excitation flash rate. Samples receiving flashes at 2 Hz have a 520 nm absorption change of approx. 50% compared to the 0.33 Hz rate (Fig. 4). This decrease is believed to be due to a decrease in the number of PS II charge separations that take place when excitation flashes are given at 2 Hz. In the +10 mM HCO_3^- samples no flash rate dependence in the amplitude of the 520 nm absorption change is observed (Fig. 4). Also, on the first flash after 5 min dark adaptation the amplitude of the 520 nm absorption change was $1.2 \cdot 10^{-3}$ for both depleted and reconstituted chloroplasts. This is consistent with the fluorescence data previously mentioned and supports the concept that HCO_3^- depletion does not cause an appreciable decrease in the number of active PS II reaction centers.

Delayed light emission after a single flash

There are a number of theories to explain the origin of delayed light emission in photosynthetic material [27 – 29]. The most widely accepted theory is that of charge recombination, which can be written schematically as follows:

$$\text{P-680-Q} \underset{h\nu_{\text{DLE}}}{\overset{h\nu}{\quad}} \text{P}^{*}\text{-680-Q} \leftrightarrows \text{P}^{+}\text{-680-Q}^{-}$$

where P^*-680 represents the PS II reaction center chlorophyll in a singlet excited state and $h\nu_{\text{DLE}}$ quanta given off as delayed light emission. Any changes in the sample that impede the stabilization of these charges will cause delayed light emission to be enhanced. Other phenomena such as membrane potential and proton gradients may also affect delayed light emission. These effects are minimized here by observing delayed light emission after a single flash [30] and by using chloroplasts that are uncoupled (see previous section).

In Fig. 5 the effect of HCO_3^- depletion on delayed light emission decay in the 150 μs to 4 s range is shown. For times greater than 300 μs after the flash the HCO_3^- depletion causes delayed light emission to decay more slowly. As expected, this is similar to the effect of DCMU since a competing process, the reoxidation of Q^-, is slowed in depleted samples [10]. The effect of depletion is smaller than that of DCMU because the block in Q^- reoxidation is not complete. For the 140–300 μs range the depleted sample has its delayed light emission inhibited by a small amount. To emphasize a direct effect of HCO_3^- depletion on delayed light emission, DCMU was added to block all forward electron transfer from Q^-. If the only effect of HCO_3^- was to speed Q^- oxidation in the forward direction, addition of DCMU should eliminate this effect, and delayed light emission should be the same in depleted and reconstituted samples. The results of this type of measurement are shown in Fig. 6. In virtually the entire time range measured, the depleted sample had a smaller amount of delayed light emission. One reason for this could be the shift in dark S-state distribution that occurs in depleted chloroplasts as shown in Table II. In depleted chloroplasts a single flash would produce less S_2, and it is known that the lower oxidized S-states give rise to greater levels of

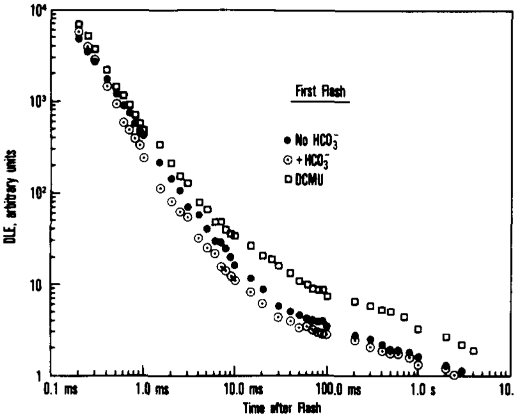

Fig. 5. The decay of delayed light emission following a single saturating excitation flash. Decays are shown for chloroplasts depleted of HCO_3^- (●), +10 mM HCO_3^- (○), and control (undepleted) with 3 μm DCMU present (□). The chloroplast concentration for these measurements was 10 μg Chl/ml.

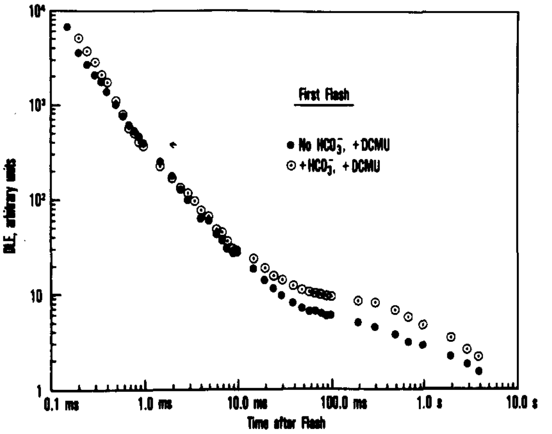

Fig. 6. The decay of delayed light emission following a single saturating excitation flash. Decays are shown for chloroplasts with 3 μM DCMU present and depleted of HCO_3^- (●) and +10 mM HCO_3^- (○). The chloroplast concentration for these measurements was 10 μg Chl/ml.

delayed light emission [31,32]. At the same time, this lower amount of delayed light emission might indicate a decrease in the probability for back-reaction by HCO_3^- effects on or near Q.

Formate effects

It was found that the presence of formate in the reaction medium, even without HCO_3^- depletion, caused a slower decay of microsecond-range fluorescence and increased the damping in oxygen-evolution flash-yield patterns. In this work we chose to study the effects of HCO_3^- without any additional effects that formate might cause. Experiments are now underway that include formate in the reaction medium. A future publication will be devoted to the findings of these investigations. It should be kept in mind that a strict comparison between the results presented here and previously published work cannot be made, since all previous investigations included formate in the reaction medium.

Discussion

The most significant observation reported here is the occurrence, in depleted chloroplasts, of a very slow fluorescence decay component, even following a single excitation flash (Fig. 1). The decay in fluorescence after the first flash corresponds to the $Q^-B \rightarrow QB^-$ reaction, and this is in the microsecond as well as in the seconds range. The effect of HCO_3^- depletion reported here is quite distinct from that on the $Q^-B^{2-}A \rightarrow QB^-A^{2-}$ reaction reported earlier [11], since the latter is only seen after the third flash and if flashes are given at a rate of 10 Hz or higher (Fig. 2A). The portion of the total variable fluorescence, *f*, after the third flash for flashes given at 1 Hz is $0.42/2.0 = 0.21$, and for flashes given at 10 Hz an additional $0.24/2.0 = 0.12$ (Fig. 2A). So the HCO_3^- depletion effect on the $Q^-B \rightarrow QB^-$ reaction is quite significant and accounts for almost twice as much variable fluorescence at 90 ms after the flash as does the effect on the $Q^-B^{2-} \rightarrow QB^-A^{2-}$ reaction.

The slow reoxidation of Q^- in depleted samples will result in a portion of reaction centers not becoming open for photochemistry between flashes if flashes are given at rates of a few hertz. This is the basis for the HCO_3^- effect on repetitive flash-excited photoreactions. Table I indicates that based on the slow fluorescence decay for flashes given at 2 Hz, one should expect an approx. 30% enhancement in flash-excitation photoreactions upon the addition of HCO_3^-. This is the approximate amount of enhancement that is observed for oxygen flash yield [8], ESR signal IIvf and absorption change at 334 nm [12]. Another prediction of this hypothesis is that the enhancement caused by HCO_3^- addition should be dependent upon excitation flash rate (see Table I). This is demonstrated to be the case for oxygen evolution (Fig. 3A–C and Table II) and for the 520 nm absorption change (Fig. 4).

The explanation that has been given previously for inhibition of flash-excitation photoreactions is that HCO_3^- depletion inactivates a portion of the PS II reaction centers [1,10,12]. This is certainly an incorrect interpretation, since it cannot explain the dependence of the amount of inhibition on flash rate. Also, as is shown in this work in dark-adapted samples, the amplitude of variable fluorescence yield and 520 nm absorption change after the first flash is unaffected by the presence or absence of HCO_3^-. From these results one would predict that if ESR signal IIvf and absorption changes at 680 and 334 nm could be measured after the first flash, they would also show no HCO_3^- effect. A HCO_3^-

effect is seen in these measurements as a change in signal amplitude only because multiple flash excitation at 1 or 2 Hz was used.

The conclusion that HCO_3^- does not directly inactivate PS II reaction centers is consistent with measurements [13,33] of the Hill reaction, which were found to be unaffected by depletion. For these measurements, the site of HCO_3^- action between Q and B was circumvented by the use of silicomolybdate [13] to accept electrons directly from Q or chloroplasts that were treated with trypsin and had ferricyanide as an acceptor [33].

A recent measurement [34] of proton release from oxygen evolution seems to conflict with the above hypothesis. Excitation flashes were given every 10 s, so Q^- would be almost completely oxidized between flashes (Fig. 1) and little effect of HCO_3^- would be expected, according to the hypothesis put forth in this work. However, their depleted samples appeared to have about half the PS II activity of the control (undepleted) sample. Unfortunately, a proper $+HCO_3^-$ control could not be measured. So the inactivation effect on the water proton release that was observed may be due just to the depletion procedure and may have nothing to do with the effect of HCO_3^- itself.

The above hypothesis also can give an explanation for the 30–50% inhibition of the Hill reaction that occurs at low light intensity in depleted chloroplasts [8,1]. Saturating light intensity occurs when the PS II reaction center receives excitons approximately every 20 ms, the time of the rate-limiting step in electron transport [15]. The 1.5-fold inhibition is observed at about 1/25th of saturating intensity [8], and this would correspond to an arrival of excitons at the reaction center every 500 ms. This 1.5-fold inhibition is in fairly good agreement with the 1.3-fold inhibition calculated from the 500 ms point of Fig. 1 and Table I. In other words, in depleted samples the very slow Q^- reoxidation is in the same time range as the exciton arrival time at the reaction center in low light. Therefore, a portion (30–50%) of the reaction centers do not recover between exciton arrivals and they are unable to perform photochemistry.

The oxygen S-state transitions are slowed down from 200–400 μs to 10 μs [8]. However, approx. 20–30% of the S-state transition time course is slowed down even further to the hundreds of millisecond and seconds range [2,8]. The millisecond and seconds range reoxidation of Q^- discussed in this work explains this very slow component of the S-state transitions that takes place in depleted chloroplasts.

It is clear that slow $Q^-B \rightarrow QB^-$ reactions exist in depleted samples. The mechanism behind these reactions is not known at present. The slow fluorescence decay in the time range of hundreds of milliseconds and seconds has been attributed to Q^- reoxidation by back-reaction with positive charges during deactivation of S_2 and S_3 in the dark [35]. Proposing a change in deactivation due to HCO_3^- depletion does not seem to be reasonable, since the portion of total variable fluorescence that is controlled by S-states is at most 0.06 [35], which is about 4-fold smaller than the amplitude of the slow fluorescence component due to HCO_3^- depletion. If the slower Q^- reoxidation in depleted samples was due to a decrease in the back-reaction with positive charge (S_2 and S_3), then addition of HCO_3^- would cause enhanced back-reaction and a loss in S-state positive charge. This is obviously not the case, because addition of HCO_3^- results in increased amounts of positive charge formation and use. Perhaps the depletion of HCO_3^- causes an increase in Q redox potential with respect to B and in the redox potential of B with respect to the plastoquinone pool. This would alter the equilibrium states of all these charge carriers and possibly slow the forward reactions $Q^-B \rightarrow QB^-$ [10] and $Q^-B^{2-}A \rightarrow QB^-A^{2-}$ [11]. The higher redox potential of Q would also make the energetics for delayed light emission less favorable. This could account for an inhibition in delayed light emission that occurs in depleted samples (Fig. 6).

Another hypothesis [36] is that HCO_3^- forms a salt bridge with an arginine residue of a protein associated with Q and B. This salt bridge gives rise to a local electric field that stabilizes the $Q^-B \rightarrow QB^-$ reaction. Depletion of HCO_3^- would reduce this field and slow this reaction. This mode of action would occur without a change in the redox potential of Q or B.

The S_0/S_1 ratio was observed to shift in HCO_3^--depleted chloroplasts (Table II). It has been shown [37] that the S-state distribution in the dark can be altered chemically by shifting the redox potential of the reaction medium. A model has been proposed by Kok et al. [38] that all the S-states are in equilibrium with oxygen and reductants that are present. It has also been shown by Greenbaum and Mauzerall [39] that other oxidants, besides oxygen, can shift the S-state distribution. Apparently, in depleted chloroplasts these equilibrium reactions are shifted to more reducing conditions. This shift in S-state distribution to a more reduced configuration is also shown by the inhibition of delayed light emission after a single flash (Fig. 6).

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