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EFFECTS OF BICARBONATE DEPLETION ON SECONDARY ACCEPTORS OF PHOTOSYSTEM II

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In thylakoid membranes incubated in the dark with ferricyanide, an auxiliary acceptor (Q400) associated with Photosystem II becomes oxidized. It has been reported that, based on oxygen flash-yield data, electron flow to Q400 did not occur in 'bicarbonate-depleted' (formate-pretreated) samples. Contrary to this earlier report, we find, based on oxygen flash-yield data and chlorophyll *a* fluorescence-transient measurements, that Q400 is active as an electron acceptor in formate-pretreated samples. It is concluded that the effect of formate pretreatment is on the flow of electrons between Q, B and the plastoquinone pool and not the flow to Q400. We also believe that another auxiliary acceptor of Photosystem II exists under conditions of formate pretreatment and pH larger than 7.0. This belief is based on increased double advancement in the oxygen flash-yield pattern and increased area above the chlorophyll *a* fluorescence-rise curve. The double advancement in the oxygen pattern shows a second-order dependence on flash intensity. These effects are eliminated by bicarbonate addition or shifts to lower values of pH such as 6.8. This new acceptor is believed to be different from Q400.

Introduction

Monovalent anions, such as formate and particularly bicarbonate, can control the rate of electron flow through Photosystem II [1–4]. The effects of bicarbonate have been studied extensively (for reviews, see Ref. 5 and 6). The binding of bicarbonate and other monovalent anions is to a specific site on the Photosystem II complex [7].

To observe an effect of added bicarbonate on electron flow rates, it is first necessary to wash chloroplasts in a low-pH buffered solution that contains a high concentration of formate. This treatment is termed the 'HCO_3^- depletion' procedure. There is now evidence [8], however, that, rather than removing endogenous HCO_3^-, the main effect of the procedure is to add an inhibitory anion (formate) to the anion binding site on Photosystem II, thus slowing electron flow. It is proposed that bicarbonate stimulates electron flow by replacing the more inhibitory formate anion. This alternative way of viewing the 'bicarbonate-effect' requires new terminology. In this work, we will use the term formate-pretreated thylakoids to refer to what have been previously called 'bicarbonate-depleted' thylakoids, i.e., those subjected to the 'bicarbonate-depletion' procedure. We will show that treating thylakoids in a low pH, high-formate medium has effects on Photosystem II not previously described.

Originally, it was hypothesized [3] that the ef-

Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Pipes, 1,4-piperazinediethanesulphonic acid.

fect of bicarbonate on Photosystem II was on the oxygen-evolving system. There are more recent reports [4,9,10] indicating that bicarbonate does affect the oxygen side of Photosystem II. However, under most conditions, the rate-limiting step affected by bicarbonate is found on the reducing side of Photosystem II [11–13]. In formate-pretreated thylakoids, addition of bicarbonate normally increases the rate of electron movement between the primary acceptor, Q, and the secondary acceptor, B [12,14]. There are reports [15–17] that the movement of electrons between B and the plastoquinone pool is also increased by added bicarbonate. Besides these changes in the kinetics of electron flow on the reducing side of Photosystem II, bicarbonate also alters herbicide sensitivity [18–20]. The primary effect of herbicide action is on electron flow on the reducing side of Photosystem II. In this report, we are investigating another effect of bicarbonate on the reducing side of Photosystem II, its relationship to auxiliary acceptors.

The primary stable acceptor of Photosystem II has been designated Q [21] and is believed to be a quinone [22] capable of accepting one electron. A number of other acceptors have also been detected. One, designated Q400, is observed at high redox potentials, normally established by ferricyanide [23]. This component is known to be a single-electron acceptor with a midpoint potential of 400 mV at pH 7 [24]. This particular acceptor has been shown to participate in double turnovers in oxygen evolution [25,26] during the first flash given in a series. Other acceptors, distinct from Q or Q400 are believed to exist and have been designated as X_a [27,28], Q2 [29] and W [30]. These acceptors are unique because they allow charge separation to occur without formation of a membrane potential or a change in the average oxygen yield. A recent report [31] suggests that Q2 can participate in double advancement in oxygen evolution when supersaturating laser-excitation is used.

It has been suggested by Radmer and Ollinger [32], based on oxygen data, that 'bicarbonate depletion' inhibited electron flow to Q400. We report here an important clarification. Our oxygen flash-yield and chlorophyll *a* fluorescence-transient measurements indicate that Q400 is active in electron flow in 'bicarbonate-depleted' samples when formate is omitted from the reaction mixture. Also, we have evidence of an additional acceptor, not Q400, that becomes oxidized and active in photochemistry only under the special conditions of formate-pretreatment and pH > 7.0. This acceptor is involved in Photosystem II charge separation and allows double advancement in oxygen evolution to take place.

Methods

Thylakoid preparation

Broken chloroplasts (thylakoids) were isolated from leaves of Dwarf peas (*Pisum sativum*) grown in a laboratory growth chamber and harvested 14 to 21 days after germination. The isolation procedure was as previously described [33]. Both freshly prepared thylakoids that were osmotically shocked and thylakoids stored under liquid nitrogen were used. The thylakoids were formate-pretreated by the low-pH 'bicarbonate-depletion' method previously described [34]. The following reaction media were used: at pH 6.8, 50 mM phosphate/200 mM NaCl and at pH 7.8, 50 mM Tricine/200 mM NaCl. In some cases, 100 mM formate was present in the reaction medium, and then the NaCl concentration was decreased to 100 mM.

Oxygen flash yield

A bare platinum electrode was used to detect yields of oxygen upon flash illumination. The platinum surface was 1.5 × 15 mm with a 0.18 mm channel depth where the sample was deposited. A sample concentration of 0.5 mg chlorophyll/ml was used, and at least 10 min was allowed for sample settling prior to taking a measurement. A sealed upper chamber was filled with N_2 bubbled reaction medium to avoid exposure of the sample for long periods of time to buffer containing CO_2 and bicarbonate. The platinum was biased at −650 mV with respect to an Ag/AgCl junction in the upper chamber. Signals were detected by a laboratory-built, DC-coupled, transimpedance amplifier that had a 2 ms risetime. Flash excitation was provided at a 1 Hz flash rate by a xenon strobe-lamp that has a 3 μs width at half height.

Chlorophyll a-fluorescence yield

The fluorescence-yield rise in the microsecond

range was measured with the single-flash method [12]. However, in this work, all data were digitized by a Biomation model 805 waveform recorder, and calculations were done with a Heath H8 minicomputer *. The chlorophyll *a*-fluorescence transient was measured as previously described [26]. Areas above the fluorescence-rise curve were determined by integration of digitized data with the minicomputer.

Results

It has been reported [25,26] that ferricyanide can increase the oxygen yield on the second flash of a series given to dark-adapted thylakoids. This observation has been interpreted as an increase in double advancement in S-states following the first flash only. Fig. 1 shows that this is the case in formate-pretreated samples when bicarbonate is added. We find, however, that ferricyanide treatment also causes double advancement to occur in oxygen S-states in formate-pretreated samples when bicarbonate is not added, provided formate is omitted from the reaction mixture. This is seen in Fig. 2 for the ferricyanide-treated sample as an increase in the yield of flash two and as a slight advancement of the flash-yield pattern easily seen for flashes 5 and 6 and 9 and 10. This is in apparent contradiction to results of Radmer and Ollinger [32], who found only a slight increase in the flash-two yield in formate-pretreated samples given ferricyanide. However, these authors had formate in their reaction mixture, a practice that greatly distorts the oxygen flash-yield pattern (Fig. 3). For such highly damped yield patterns in the presence of formate, it is difficult to determine if double advancement in S-states is being altered. We can conclude, though, that it is not the absence of bicarbonate that prevents double advancement in ferricyanide-treated thylakoids, but most likely the presence of formate.

Upon continuous illumination of chloroplasts that have been kept in the dark for 5 min or longer, the chlorophyll *a* fluorescence increases [35]. This rise in fluorescence has been interpreted as due to a reduction by Photosystem II of a fluorescence quencher, Q, to a nonquenching form, Q^- [21]. For chloroplasts with diuron present, a linear relationship exists [36] between variable fluorescence and photochemical rate. Thus, if the same maximum fluorescence level is attained un-

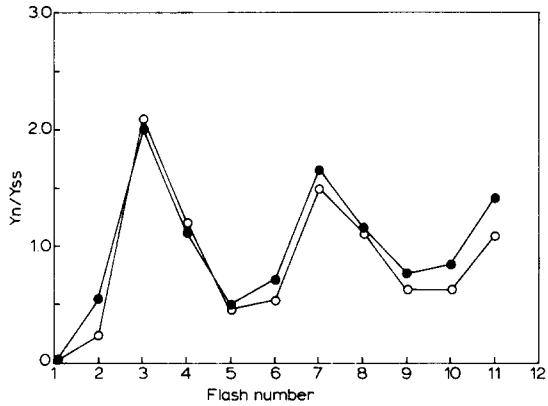

Fig. 1. Oxygen flash yield as a function of flash number in formate-pretreated thylakoids that were reconstituted by addition of 10 mM bicarbonate. The samples then had no other additions (○——○) or were incubated at 20°C at a concentration of 5 mg chlorophyll/ml for at least 10 min with 1.5 mM ferricyanide (●——●). The thylakoids were diluted 10-fold with formate-free reaction medium at pH 6.8 that included 1 mM NADP prior to placement on the electrode. The oxygen yield on any flash, Y*n*, is normalized by the steady-state yield, Yss, reached after 22 flashes.

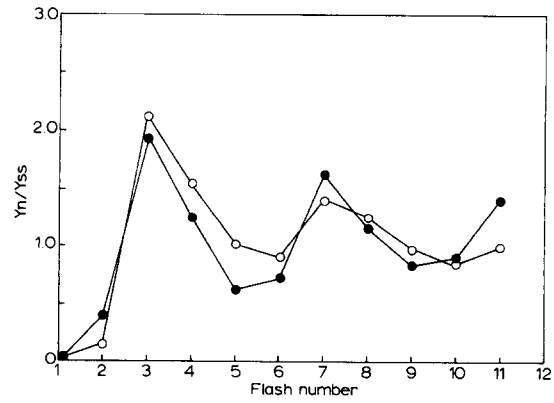

Fig. 2. Oxygen flash yield as a function of flash number in formate-pretreated thylakoids. All other conditions are as in Fig. 1.

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

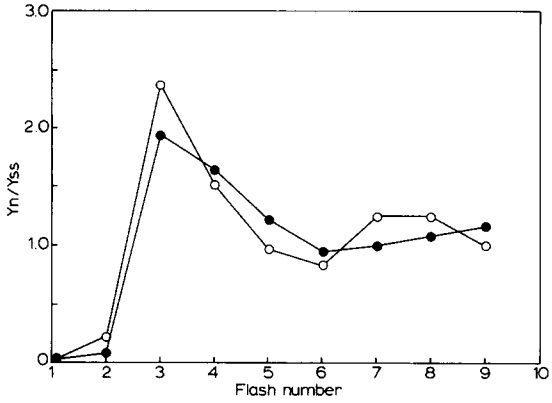

Fig. 3. Oxygen flash yield as a function of flash number in formate-pretreated thylakoids. The thylakoids were not incubated with ferricyanide but were suspended in pH 6.8 reaction medium that did (○——○) or did not (●——●) include 100 μM formate. When formate was not present, 100 mM NaCl was included to maintain the same ionic strength. All other conditions are as in Fig. 1. The steady-state yield in samples containing formate was 70% of that in samples without formate.

der different conditions [37], the areas above the fluorescence-rise curve is proportional to the quencher (electron acceptor) concentration.

Ikegami and Katoh [23] have demonstrated that the normalized area above the fluorescence curve increased 100% upon incubation with ferricyanide prior to the addition of diuron. They suggested that an acceptor with a redox potential of 360 mV at pH 7.8 was oxidized in the dark by ferricyanide. It is this auxiliary acceptor that participates in multiple-charge separation at the Photosystem II reaction center on the first flash and the consequent double advancement in S-states [26]. If the increased yield on flash 2 in Fig. 2 is due to ferricyanide, oxidizing an auxiliary acceptor in formate-pretreated thylakoids, then an increase in area above the fluorescence-rise curve can be expected. Also, if multiple charge-separations occur at the reaction center, then this additional area should be partially or wholly eliminated by a single preillumination flash. In other words, multiple charge-separations will reduce both Q and an auxiliary acceptor.

The effects of incubation with ferricyanide on the area above the fluorescence-rise curve are presented in Table I for control (not formate-pretreated), for just formate-pretreated and for formate-pretreated, bicarbonate-added thylakoids. In control samples, ferricyanide causes the area above the fluorescence-rise curve to increase by $(3.04/4.56) \times 100 = 66\%$, which is in good agreement with previous reports [23,26]. The area above the rise-curve increases by $(1.80/3.84) \times 100 = 47\%$ and $(2.54/5.11 \times 100) = 50\%$ for just formate-pretreated and for formate-pretreated, bicarbonate-added samples, respectively. This indicates that ferricyanide can oxidize an auxiliary acceptor (Q400) in formate-pretreated as well as in bicarbonate-added and control samples.

As anticipated, this auxiliary acceptor is partially reduced by a single preillumination flash. The data in Table I show that 45, 28 and 36% of the additional area can be eliminated by a single flash in control, formate-pretreated, and bicarbonated-added thylakoids, respectively. In making these calculations, it is presumed that a preillumination flash reduces Q first and then any auxiliary acceptor. However, it should be kept in mind that the measurements made here can in no way distinguish between area due to Q or to an auxiliary acceptor. These data, in combination with the results shown in Fig. 2, indicate that this auxiliary acceptor can take part in Photosystem II charge separation and oxygen evolution in formate-pretreated ('bicarbonate-depleted') thylakoids.

While doing these experiments, we found other conditions, which do not require ferricyanide, when double advancement in oxygen evolution occurs. In formate-pretreated chloroplasts suspended in formate-free reaction mixture at pH 7.8, significant double advancement in the oxygen pattern occurs as shown in Fig. 4 by an enhanced yield on flash two. This does not occur in similarly treated chloroplasts at pH 6.8. Neither does it occur at pH 7.8 if the chloroplasts are resuspended in reaction mixture that contains 10 mM bicarbonate (Fig. 1). Fig. 5 shows how double advancement in the oxygen pattern changes with pH in formate-pretreated thylakoids. A rather dramatic increase in double advancement begins to occur at pH values greater than 7.0. To obtain large yields on flash two as shown in Fig. 4, it was best to use freshly prepared thylakoids that had been kept on ice for only a few hours at most. Such sensitivity to

TABLE I

AREA ABOVE THE CHLOROPHYLL *a* FLUORESCENCE-RISE CURVES OF THYLAKOIDS WITH DIURON PRESENT AND INCUBATED WITH FERRICYANIDE

Thylakoids at a concentration of 10 μg chlorophyll/ml were incubated at 20°C with 0.2 mM ferricyanide for 5 min in the dark, prior to addition of 10 μM diuron. 1 min after the addition of diuron, the measurement was made. The values are normalized by $\Delta F = F_{max} - F_0$; where F_0 is the fluorescence level, the instant illumination has begun and F_{max} is the maximum level of the fluorescence rise. The additional area is the increase in area due to ferricyanide incubation or decrease in area due to a preillumination flash. The preillumination flash was given 75 ms prior to the start of continuous illumination.

Sample conditions	Area/ΔF	Additional area	Area eliminated by flash	Additional area eliminated by flash
Control (not pretreated)				
No ferricyanide ($F_0 = 22$, $F_{max} = 64$)	4.56	–	–	–
No ferricyanide ($F_0 = 22$, $F_{max} = 64$) and one preillumination flash	1.43	–	4.56 – 1.43 = 3.13	–
+ Ferricyanide ($F_0 = 20$, $F_{max} = 47$)	7.60	7.60 – 4.56 = 3.04	–	–
+ Ferricyanide ($F_0 = 20$, $F_{max} = 47$) and one preillumination flash	3.10	–	7.60 – 3.10 = 4.50	4.50 – 3.13 = 1.37 or 1.37/3.04 = 0.45
Formate-pretreated				
No ferricyanide ($F_0 = 36$, $F_{max} = 77$)	3.84	–	–	–
No ferricyanide ($F_0 = 36$, $F_{max} = 77$) and one preillumination flash	1.04	–	3.84 – 1.04 = 2.80	–
+ Ferricyanide ($F_0 = 28$, $F_{max} = 64$)	5.64	5.64 – 3.84 = 1.80	–	–
+ Ferricyanide ($F_0 = 28$, $F_{max} = 64$) and one preillumination flash	2.34	–	5.64 – 2.34 = 3.30	3.30 – 2.80 = 0.50 or 0.50/1.80 = 0.28
Formate-pretreated and bicarbonate-added				
No ferricyanide ($F_0 = 33$, $F_{max} = 71$)	5.11	–	–	–
No ferricyanide ($F_0 = 33$, $F_{max} = 71$) and one preillumination flash	1.71	–	5.11 – 1.71 = 3.40	–
+ Ferricyanide ($F_0 = 29$, $F_{max} = 56$)	7.65	7.65 – 5.11 = 2.54	–	–
+ Ferricyanide ($F_0 = 29$, $F_{max} = 56$) and one preillumination flash	3.33	–	7.65 – 3.33 = 4.32	4.32 – 3.40 = 0.92 or 0.92/2.54 = 0.36

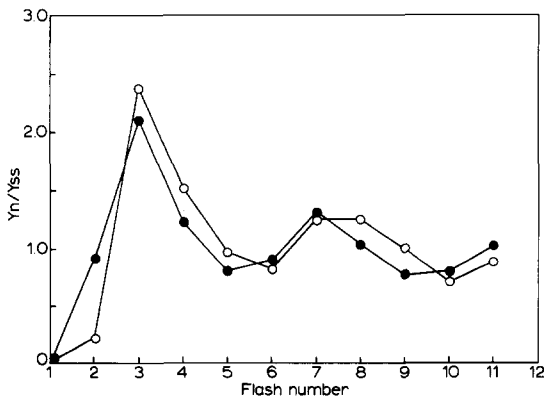

Fig. 4. Oxygen flash yield as a function of flash number in formate-pretreated thylakoids without ferricyanide being present. Samples were suspended in formate-free reaction medium at pH 6.8 (○——○) or pH 7.8 (●——●). All other conditions are as in Fig. 1.

sample treatment has been reported previously [26].

If the increase in the oxygen yield on flash two is due to double advancement in depleted thylakoids at pH 7.8, then the light curve should follow a multiple-hit Poisson distribution: $P = 1 - (1 + \sigma I)e^{-\sigma I}$, where P is the probability of occurrence, I is the flash intensity, and σ is the optical cross-section. The oxygen yield at steady state is due to one advancement in the S-states and should follow a one-hit Poisson distribution: $P = 1 - e^{-\sigma I}$. Double hits are not a consideration in the steady-state measurements, because any secondary acceptor becomes reduced after the first couple of flashes. Fig. 6 is a measurement of light curves for the steady-state yield and the yield on flash two.

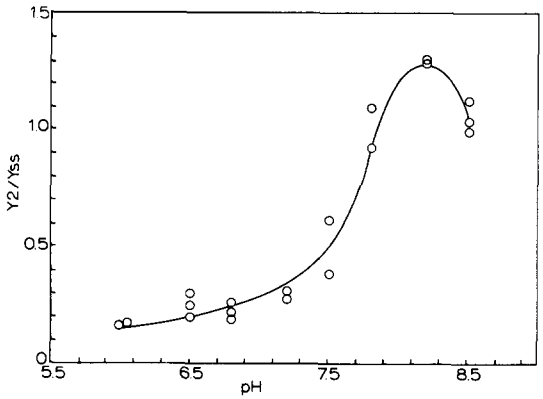

Fig. 5. Oxygen yields on flash two (Y2) as a function of assay medium pH. The oxygen yields were normalized by Yss, the steady-state yield reached after 22 flashes. The thylakoids were pretreated with formate and were not incubated with ferricyanide. In the pH range of 8.5–7.5 20 mM Tricine was used, and in the range of 7.2–6.0 20 mM Pipes was used as a buffer. In all ranges, 200 mM NaCl was present as an electrolyte.

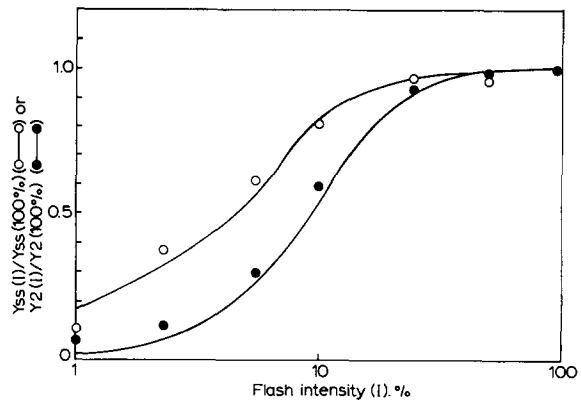

Fig. 6. Light curves for the oxygen flash yield at steady state (Yss) or after the second flash (Y2). Yss at full intensity, Yss(100%), was measured on flash 22. The flash intensity was then decreased by insertion of an appropriate neutral-density filter and Yss(I) was measured on flash 23. Y2 at full intensity, Y2(100%), was measured with no flash attenuation. Y2(I) was measured with only the first-flash intensity decreased to the desired level. Thylakoids were formate-pretreated and suspended in formate-free reaction medium at pH 7.8, and NADP was present at a concentration of 1 mM as an electron acceptor. Theoretical curves for a one-hit (○——○) and multiple-hit (●——●) Poisson distribution are drawn with $\sigma = 1/5.5\%$.

The expected correlation to single- and multiple-hit light curves is found, and this supports the hypothesis that double turnovers in Photosystem II

TABLE II

AREA ABOVE THE CHLOROPHYLL *a* FLUORESCENCE-RISE CURVES OF THYLAKOIDS WITH DIURON PRESENT AND NO FERRICYANIDE

Thylakoids were suspended in formate-free reaction medium of pH 6.8 or 7.8. Reconstituted samples had 10 mM bicarbonate added to them. All other conditions are as in Table I.

Sample conditions	Area/ΔF	Additional area	Area eliminated by flash	Additional area eliminated by flash
Formate-pretreated				
pH 6.8 ($F_0 = 20$, $F_{max} = 81.5$)	1.29	–	–	–
pH 6.8 ($F_0 = 20$, $F_{max} = 81.5$) and one preillumination flash	0.39	–	1.29 – 0.39 = 0.90	–
pH 7.8 ($F_0 = 20$, $F_{max} = 73.5$)	2.34	2.34 – 1.29 = 1.05	–	–
pH 7.8 ($F_0 = 20$, $F_{max} = 73.5$) and one preillumination flash	0.87	–	2.34 – 0.87 = 1.47	1.47 – 0.90 = 0.57 or 0.57/1.05 = 0.54
pH 7.8 ($F_0 = 16$, $F_{max} = 60.0$) + 0.2 mM ferricyanide	4.29	4.29 – 2.34 = 1.95	–	–
Formate-pretreated and bicarbonate-added				
pH 6.8 ($F_0 = 20$, $F_{max} = 75$)	1.28	–	–	–
pH 6.8 ($F_0 = 20$, $F_{max} = 75$) and one preillumination flash	0.49	–	1.28 – 0.49 = 0.79 or 0.79/1.28 = 0.62	–
pH 7.8 ($F_0 = 20$, $F_{max} = 74$)	1.32	1.32 – 1.28 = 0.04	–	–
pH 7.8 ($F_0 = 20$, $F_{max} = 74$) and one preillumination flash	0.32	–	1.32 – 0.32 = 1.00 or 1.00/1.32 = 0.76	–

underlie the increased oxygen yield on flash two seen in Fig. 4.

To demonstrate if this advancement in oxygen yield corresponds with the presence of an auxiliary acceptor besides Q, the area above the fluorescence-rise curve with diuron present was determined. The results are shown in Table II. In formate-pretreated thylakoids suspended in formate-free reaction mixture at pH 7.8, an 81% increase in the area was observed compared to similarly treated thylakoids at pH 6.8. In bicarbonate-added samples, essentially no change in the area occurred at either of these pH values. Apparently, under the special conditions of formate-pretreatment and pH 7.8, an auxiliary electron acceptor becomes oxidized.

We were interested in finding out whether the acceptor oxidized by ferricyanide (Q400) and the acceptor oxidized in formate-pretreated thylakoids were different. If these acceptors were different one would expect additive effects when they were both oxidized. Simultaneous oxidation was accomplished by adding appropriate amounts of ferricyanide to thylakoids that had been formate-pretreated and resuspended in pH-7.8 reaction medium. The oxygen flash-yield pattern was found to be identical (data not shown) to that of formate-pretreated thylakoids at pH 7.8, as seen in Fig. 4. The area above the chlorophyll *a* fluorescence transient was increased from 2.34 to 4.29 by the inclusion of ferricyanide (see Table II). Based on this additive nature of the transient data, these acceptors indeed appear to be different. The absence of change in the oxygen flash-pattern, when both acceptors were oxidized, must be due to kinetic limitations in the oxygen involving process that restrict the amount of double hitting that can take place.

Another indication of auxiliary acceptors being present is a delay or slowing of chlorophyll *a* fluorescence rise during a flash of a few microseconds duration. Normally, if only one acceptor is available, and care is taken to avoid large-scale triplet formation, the fluorescence rise during a flash is limited in time by the rate of one-hit quanta arrival [38]. However, if two acceptors are available, then the rise is limited by the rate of two-hit quanta arrival, and the fluorescence rise will appear to be delayed or slowed. This phenomenon was observed [24] for samples incubated with ferricyanide. Fig. 7 shows that this delayed and slowed fluorescence rise is observed on the first flash for formate-pretreated thylakoids at pH 7.8, but is not seen when bicarbonate is added to the reaction mixture.

The fluorescence rise is much more alike for the formate-pretreated and bicarbonate-added thylakoids during the second flash when the auxiliary acceptor has been largely reduced. These data are quite consistent with two acceptors being available in dark-adapted formate-pretreated and bicarbonate-added samples at pH 7.8. It should also be noted that for formate-pretreated and bicarbonate-added samples at pH 6.8, the fluorescence rise is identical (data not shown), in agreement with earlier findings [12]. This points out again the special conditions of formate-pretreatment and pH 7.8 needed for this auxiliary acceptor to be oxidized and functional in charge separation.

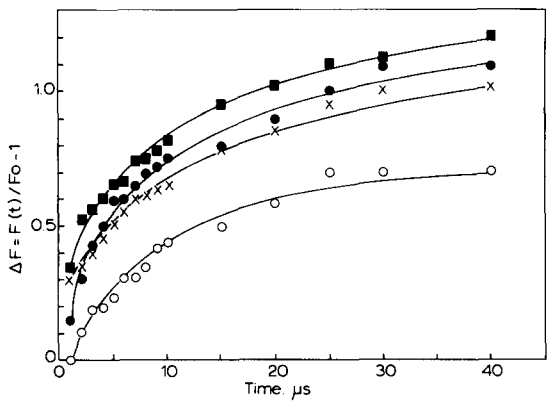

Fig. 7. Variable chlorophyll *a* fluorescence (ΔF) rise during an excitation flash. The excitation-flash intensity was adjusted to minimize the generation of triplet quenchers. This was accomplished by the use of a flash intensity low enough to avoid a dip in the rise in fluorescence from occurring at the flash peak at 3 μs. $\Delta F = (F(t) - F_0)/F_0$, where $F(t)$ is the fluorescence at any time during the flash and F_0 is the fluorescence level observed in the dark-adapted sample. The fluorescence rise is shown for the first flash in formate-pretreated (O——O) and reconstituted (●——●) thylakoids. It is also shown for the second flash in formate-pretreated (×——×) and reconstituted (■——■) thylakoids. Samples were at a chlorophyll concentration of 10 μg/ml in formate-free reaction medium at pH 7.8. Samples were reconstituted by addition of 10 mM bicarbonate.

Discussion

It is now clear that high concentrations of monovalent anions given to chloroplast thylakoids slow the rate of electron flow between Q and B [11–13], and hence to the plastoquinone pool. Substitution of the bound monovalent ion by bicarbonate under most conditions increases the rate of electron movement. However, the effects of anions on auxiliary Photosystem II acceptors are less clear; the results presented here allow additional conclusions.

At pH 7.8, formate-pretreated samples show evidence of another acceptor being present. This acceptor allows double advancement in oxygen S-states (Fig. 4) when adequate flash intensities are used (Fig. 6). One interesting aspect of photochemistry that involves this acceptor is the large amount of double advancement that does occur. The yields on flash two are much greater for the depletion-pH 7.8 acceptor (Fig. 4) than they are for Q400 (Figs. 1 and 2). However, amounts of additional area in the fluorescence-rise curves, which are indicative of the amount of auxiliary acceptor, are not all that different for the two cases. The formate-pretreated thylakoids at pH 7.8 have a $(1.05/1.29) \times 100 = 81\%$ increase (Table II), whereas the ferricyanide-treated samples have a $(3.04/4.56) \times 100 = 67\%$ increase (Table I). Also, the amount of auxiliary acceptor, eliminated by a single flash, is 45% in ferricyanide-treated samples (Table I) and 54% in formate-pretreated samples at pH 7.8 (Table II). While these values are not that much different, the resultant increase in oxygen yield on flash two is much larger for the formate-pretreated pH 7.8 case. We suggest that this can be explained by an effect of formate pretreatment on the oxygen side of Photosystem II. Under formate-pretreatment conditions, the additional positive charge, that is generated by double charge-separation at the reaction center, can be used more efficiently for advancement in S-states than in the reconstituted case (bicarbonate-containing samples) treated with ferricyanide. This is consistent with the observations that under proper conditions, bicarbonate can inhibit oxygen evolution under continuous illumination [4] and enhance delayed light emission [14] after a single flash. As already suggested [4], there are two effects of bicarbonate. It is needed for electron flow from Q to B and from B to the plastoquinone pool to operate at a high rate. This is the usual enhancement effect. It also can inhibit Photosystem II reactions, as do other anions [4]. This latter effect is observed in this work as a low portion of double S-state advancement in reconstituted samples incubated with ferricyanide, compared to depleted samples at pH 7.8.

The relationship of this new acceptor, observed in formate-pretreated thylakoids at pH 7.8, to other identified auxiliary acceptors X_a [27,28], Q2 [29] and W [30], has not been precisely established in this work. However, this acceptor seems to be distinct from Q400, since it becomes oxidized without ferricyanide treatment and only in formate-pretreated samples brought to pH larger than 7.0. Also, both acceptors can be oxidized simultaneously, which results in additive changes in the chlorophyll *a* fluorescence transient. The role this or any of these acceptors may play in normal physiological processes is unknown. Only through more detailed studies will the complex interaction of these acceptors with Photosystem II be revealed.

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