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EVIDENCE FROM THERMOLUMINESCENCE FOR BICARBONATE ACTION ON THE RECOMBINATION REACTIONS INVOLVING THE SECONDARY QUINONE ELECTRON ACCEPTOR OF PHOTOSYSTEM II

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In this paper, we present the first measurements on thermoluminescence from isolated thylakoids to probe the recombination reactions of S_2 (or possibly S_3) with Q_B^- or Q_A^-, after bicarbonate depletion and its readdition. The effects of bicarbonate depletion on the $S_2Q_B^-$ (or $S_3O_B^-$) thermoluminescence band was (1) a 6–10°C shift to a higher temperature; (2) a reduction in its intensity upon prolonged depletion; and (3) elimination after the first few flashes of the characteristic period four oscillations in its intensity as a function of the flash number. On the other hand, addition of diuron (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea), which blocks electron flow from Q_A^- to Q_B, produced the same thermoluminescence band, at about +20°C, assigned to $S_2Q_A^-$ recombination, in both depleted and reconstituted samples. These results suggest (1) the initial effect of bicarbonate depletion is to increase the activation energy for $S_2(S_3)Q_B^-$ recombination; (2) with further depletion, the incidence of this recombination decreases and the cycling of the $S_2Q_B^-$ and $S_3Q_B^-$ recombination is inhibited through effects at the Q_B apoprotein; and (3) the depletion effects are fully reversible. It is suggested that a conformational change of the PS II complex in the region of the Q_B apoprotein is responsible for these effects.

Introduction

Upon light absorption, Photosystem II (PS II) of plants leads to the oxidation of water to molecular O_2, and to the reduction of plastoquinone to plastoquinol. Electron transport in PS II may be represented as follows (for original references, see Ref. 1):

$$\begin{array}{l} \qquad\qquad\quad h\nu \\ \qquad\qquad\quad \downarrow \\ H_2O \rightarrow S_n \rightarrow Z \rightarrow \text{P-680} \rightarrow \text{Pheo} \rightarrow Q_A \rightarrow Q_B \rightarrow PQ \\ \downarrow \\ O_2 \end{array} \qquad (1)$$

where, S_n ($n = 0, 1, 2, 3$ or 4) stands for the state of charge-accumulating manganese-containing O_2-evolving system; Z, suggested to be a plastoquinol, is the first electron donor to P-680$^+$; P-680, the reaction center chlorophyll (Chl) *a*, is the primary electron donor of PS II; Pheo, pheophytin, is the primary electron acceptor of PS II; Q_A is the first bound-plastoquinone electron acceptor; Q_B is the second bound-plastoquinone electron acceptor; and PQ is the plastoquinone pool. Two

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Abbreviations: PS II, Photosystem II; Chl *a*, chlorophyll *a*; Pheo, pheophytin; PQ, plastoquinone pool; Q_A and Q_B, the first and second bound-plastoquinone electron acceptor.

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negative charges accumulate on Q_B [2,3] before the latter exchanges with a PQ molecule [4]. On the electron donor side, four positive charges must accumulate [5] before a O_2 molecule is evolved. In dark-adapted chloroplasts, the ratio of $S_0 : S_1 : S_2 : S_3 : S_4$ is $1:3:0:0:0$. Thus, the major reactions, following the first ($^1h\nu$) and second ($^2h\nu$) flashes, may be represented as:

$$S_1Q_AQ_B \xrightarrow{^1h\nu} S_2Q_A^-Q_B \rightarrow S_2Q_AQ_B^- \xrightarrow{^2h\nu} S_3Q_A^-Q_B^-$$

$$\rightarrow S_3Q_AQ_B^{2-} \rightarrow S_3Q_AQ_B \qquad (2)$$
$$PQ + 2H^+ \quad PQH_2$$

In addition to its role as the ultimate electron acceptor of photosynthesis, CO_2 (or HCO_3^-) affects photophosphorylation [6] and possibly a back reaction around PS II [7] in non-HCO_3^--depleted chloroplasts. In HCO_3^--depleted thylakoids, however, a unique stimulation of electron flow from Q_A^- to PQ pool has been observed upon HCO_3^--reconstitution [8]. Bicarbonate depletion appears to affect both the electron flow from Q_A^- to Q_B [9–11] and the electron transfer from Q_B^{2-} to the PQ pool [12]. It seems that HCO_3^- is necessary as an allosteric effector of the Q_B-protein [8,13] to which herbicides (e.g., Ref. 14) as well as quinones bind (e.g., Ref. 15). Bicarbonate depletion leads to a lowered binding of ^{14}C-herbicides [16,17]. On the basis of current models of electron transfer (see above), it has been suggested that a HCO_3^--induced conformational change allows efficient electron flow from Q_A^- to Q_B as well as efficient exchange of Q_B^{2-} with PQ [e.g., Ref. 18]. In most of the experiments on HCO_3^--depleted samples, formate (HCO_2^-), which is chemically similar to HCO_3^-, is added to assist removal of the native HCO_3^-. However, it has been shown that when the formate concentration is reduced, less [HCO_3^-] is required to observe the same stimulatory effect on the Hill reaction [8]. Thus, a competitive interaction between formate and HCO_3^- probably exists. It must be, however, emphasized that all observations, reported here, on the HCO_3^- effect are in the presence of HCO_2^-. For reviews on the HCO_3^- effect, see Refs. 13 and 19–21. Although it is now clear that, at least in the presence of HCO_2^-, HCO_3^--depletion inhibits the forward electron flow between Q_A^- and PQ, it is unclear whether or not the recombination reactions (e.g., $S_2Q_B^- \rightarrow S_1Q_B$) in PS II are also inhibited or accelerated by this treatment. Such information is necessary to understand the molecular mechanism of how HCO_3^- 'affects' electron flow in PS II.

Thermoluminescence, a phenomenon discovered by Arnold and Sherwood (Ref. 22; reviewed in Ref. 23), reflects mainly PS II reactions [24–26] and has proved to be a useful technique to probe these reactions in thylakoids [24] and leaves [27,28]. A flash-induced thermoluminescence band at 25–30 °C (the so-called B band) is suggested to be due to $S_{2(3)}Q_B^-$-recombination [24], and a flash-induced thermoluminescence band around 10 °C (the so-called D band) to be due to $S_2Q_A^-$ [24] and $S_3Q_A^-$ recombination [24,29]. The origin of the band emitted at −10 °C (the socalled A band) is not clear, although many diverse suggestions have been made in the literature (see, e.g., Refs. 23 and 26). Recent work points to the idea that it may arise from recombination of charges located on Z^+ and an acceptor beyond Q_A [30,31]. A −120 °C band (the so-called Z band) is present in isolated thylakoids as well as chlorophyll *a* in vitro, and has been suggested to originate in the triplet state [32]. The exact location of these bands depends, among other things, on the rate of heating of the sample during measurement (see, e.g., Refs. 23 and 33).

In this paper, we present results on the effect of HCO_3^- depletion, by the new continuous-depletion method, on the thermoluminescence bands to clarify our understanding of the action of HCO_3^- on the recombination reactions of $S_{2(3)}Q_B^-$ and of $S_2Q_A^-$, as well as on the Q_A^- to PQ electron transport reactions.

Materials and Methods

Thylakoid preparation

Chloroplasts were isolated from fresh market spinach and broken by osmotic shock (cf. Ref. 34). They were used either fresh or were quickly frozen at 77 K and then stored at −80 °C at a chlorophyll concentration of 2 mg/ml. The suspension medium used during storage contained 300 mM sorbitol/50 mM sodium phosphate/100 mM NaCl/5 mM $MgCl_2$ (pH 6.8).

Bicarbonate depletion

HCO_3^--free media and tubes were obtained by bubbling and flushing, respectively, with N_2 gas passed through a column containing ascarite and soda lime. HCO_3^--depletion was done by one of two methods. In the first method, the thylakoids that were washed once were obtained by a slight modification of a method previously reported [35]; it involved thawing and suspending thylakoids in HCO_3^--free depletion medium under an N_2 atmosphere at a [Chl] of approx. 30 μg/ml. In this method, the HCO_3^--depletion medium consisted of 50 mM sodium phosphate/25 mM sodium formate/100 mM NaCl/5 mM $MgCl_2$ (pH 5.8). Thylakoids were incubated for 10 min in the dark at 0 °C and pelleted (5 min at $1000 \times g$). If thoroughly HCO_3^--depleted thylakoids were required, the pellets were washed again in the depletion medium (twice washed thylakoids). The thylakoid pellet was resuspended in HCO_3^--free medium consisting of 50 mM sodium phosphate/25 mM sodium formate/100 mM NaCl/5 mM $MgCl_2$ (pH 6.5 or 6.8). In the second and a new method of HCO_3^- depletion, which was first developed by W.F.J. Vermaas [36], the experimental protocol of J.F.H. Snel, W.F.J. Vermaas and J.J.S. van Rensen (personal communication) was used. In this method (continuous depletion method), the HCO_3^--depletion medium contained 300 mM sorbitol/25 mM sodium-formate/10 mM sodium-phosphate/10 mM NaCl/5 mM $MgCl_2$ (pH 5.8). To 3.5 ml of this medium, 0.5 ml of the thylakoid suspension was added, to give a [Chl] of 250 μg/ml. Thylakoids were incubated in this medium in the dark, under CO_2-free, but H_2O-laden, N_2 atmosphere and at room temperature until we obtained the desired HCO_3^- depletion. Control samples were treated the same way except that they contained, in addition, 10 mM HCO_3^-, and were flushed with CO_2-sufficient N_2 atmosphere. Measurements were made in a HCO_3^--free reaction medium containing 300 mM sorbitol/25 mM sodium formate/25 mM sodium phosphate/10 mM NaCl/5 mM $MgCl_2$ (pH 6.5 or 6.8). In several experiments, sodium formate was left out of the reaction medium, and HCO_3^--depletion was still maintained if care was taken to keep the sample under CO_2-free N_2 atmosphere.

Thermoluminescence measurements

Thermoluminescence was measured in an apparatus similar, in principle, to that described earlier [37]. The sample holder, equipped with a heater and a thermocouple, was placed inside a Dewar flask. Luminescence was passed through a 30 Hz mechanical chopper and a red filter (Toshiba, VR-63) and focussed on a EMI 9659QB photomultiplier that was cooled from −60 to −80 °C. The photomultiplier was operated at −1.5 kV, and the signal was measured by a photoelectron counter (Digital lock-in type, model no. KC-200, JASCO, Japan). The signal was recorded on an *X-Y* recorder, with the *X*-axis driven by the analog output of a digital thermometer (TR-2112, Takeda, Riken, Japan) connected to the thermocouple on the sample holder, and the *Y*-axis by the photon counter. The accumulation rate of the counter was 16 (i.e., one analog output every 0.5 s). Reproducible measurements were obtained when 100 μl of the sample was placed homogeneously on a 2 cm × 2 cm filter paper which was held firmly in place on the sample holder by a plastic cover. The samples were handled in dim green light. The heating rate was usually 0.3 °C/s. Flash and continuous-light experiments were conducted as described earlier [24], with the difference that an orange filter ($\lambda \geqslant 570$ nm) was used in continuous-light experiments. Other details are described under Results and Discussion.

Results and Discussion

Continuous-light experiments

After thylakoids were illuminated with saturating continuous orange light during cooling to −196 °C (approx. time, 1 min), three major thermoluminescence bands were observed (Fig. 1, curve 1) with peak locations around −120 °C (Z), −20 °C (A), and +30 °C (B), their exact locations being dependent upon the rate of heating during thermoluminescence measurements.

When thylakoids were depleted of HCO_3^- by the one-wash method [35], the first observed effect was the shift of the +30 °C (B) band to a higher temperature, 40 °C (cf. curves 1 and 2, Fig. 1). It is interesting to point out that dibromothymoquinone (DBMIB), that blocks electron flow from the reduced plastoquinone pool to the cyto-

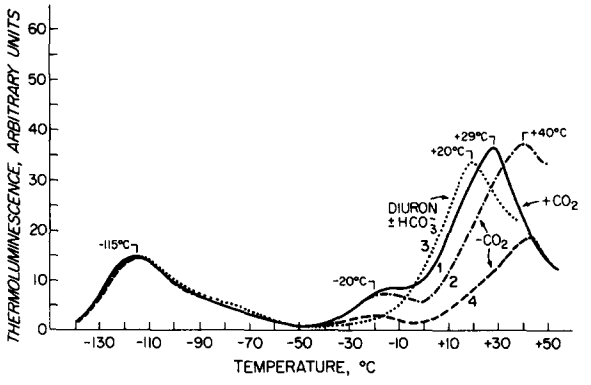

Fig. 1. Thermoluminescence of spinach thylakoids as a function of temperature. Preillumination, saturating continuous orange light during cooling to −196 °C. 1 (solid line), CO_2-(HCO_3^-)depleted sample reconstituted with 1 mM HCO_3^-; 2 (dashes and dots), sample depleted of $CO_2(HCO_3^-)$ by the one-wash method (see text); 3 (dots), reconstituted sample with 1 μM diuron – the curve for CO_2-depleted sample lay on top of this curve; 4 (dashes), sample depleted of $CO_2(HCO_3^-)$ by the twice-washed method (see text). The temperature was calibrated in the 0–50 °C range, and the markings at the lower temperatures are extrapolated from the same.

chrome $b_{6/f}$ complex, also shifts this band to a higher temperature [25]; the nature of these coincident observations needs to be further explored.

Prior to illumination, the addition of 1 μM diuron abolished the −20 °C (A) band, and shifted the +30 °C (+HCO_3^-) (or 40 °C; −HCO_3^-) (B) band to between +10° and +20 °C (curve 3, Fig. 1, Table I). This shows that the +10° to +20 °C (D) band, due to $S_2Q_A^-$ recombination, is not significantly affected by HCO_3^- depletion and confirms that the major site of action of HCO_3^- is after Q_A on the acceptor side of PS II.

TABLE I

EFFECT OF DIURON TREATMENT ON THE LOCATION OF THE THERMOLUMINESCENCE PEAKS

	Temperature of thermoluminescence Peaks (°C)			
	CO_2-depleted sample		Reconstituted (R) or Control (C) samples	
	Untreated	+Diuron (1 μM)	Untreated	+Diuron (1 μM)
Expt. 1	40	20	30 (R)	25
Expt. 2	37	15	28 (R)	12
Expt. 3	40	13	30 (C)	10

If the thylakoids, used for curve 2 in Fig. 1, were washed again in the HCO_3^--depletion buffer, the −20 °C (A) band as well as the 40 °C (B) band decreased in intensity by half (see curve 4, Fig. 1). The addition of 1 mM HCO_3^- to a HCO_3^--depleted sample restored the thermoluminescence bands to those observed in the original controls (curve 1, Fig. 1). Addition of 10 mM HCO_3^- to a HCO_3^--depleted sample gave similar results (data not shown). There was no effect of HCO_3^- on the −120 °C (Z) band.

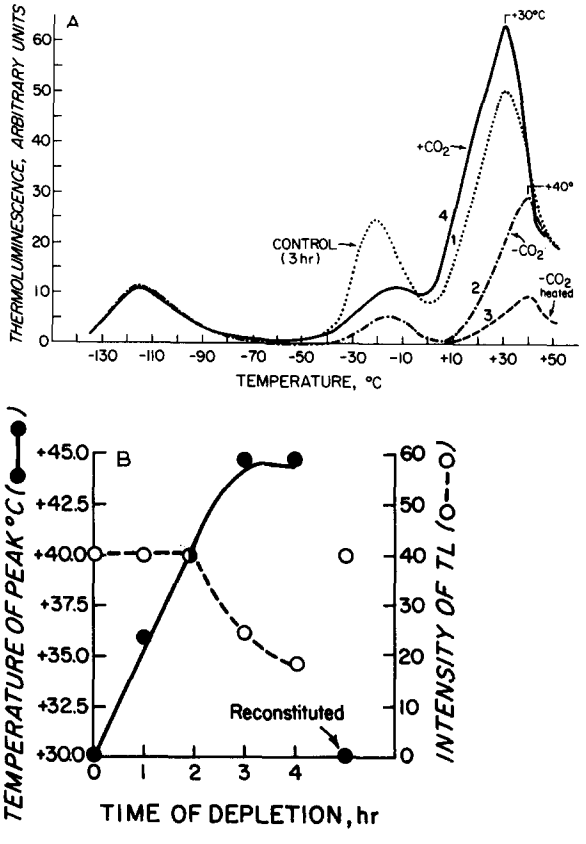

Fig. 2.(A).Thermoluminescence of spinach thylakoids as a function of temperature. Preillumination, saturating continuous orange light during cooling to −196 °C, 1 (dots), non-depleted control kept for 3 h; 2 (dashes and dots), sample depleted of $CO_2(HCO_3^-)$ by the continuous-wash method (see text); 3 (dashes), $CO_2(HCO_3^-)$-depleted sample heated at 40 °C for 10 min; 4 (solid line), $CO_2(HCO_3^-)$-depleted sample reconstituted with 10 mM HCO_3^-. (B). Temperature of the B band (●——●) and the intensity of the B band (○——○) as a function of the time of depletion by the continuous-depletion method. The data for the 'reconstituted' sample is for a 2 h depleted sample to which 10 mM HOC_3^- was added.

A similar effect was observed when HCO_3^--depletion was conducted by the continuous depletion method (Fig. 2). Data for the control sample stored for 3 h is shown in curve 1 (Fig. 2). Upon HCO_3^--depletion, a shift from 30 to 40 °C was observed (curve 2, Fig. 2). Heating thylakoids (at 40 °C for 10 min) in the absence of HCO_3^- drastically decreased the intensity of the 40 °C band without significant effects on the −20 °C band (curve 3, Fig. 2). Addition of 10 mM HCO_3^- to the sample, used for curve 2, restored the +40 °C band to the original control value (+30 °C) even 3 h after HCO_3^- depletion (curve 4, Fig. 2). Fresh control thylakoids showed similar results (data not shown). It appears that HCO_3^- depletion caused two effects. The first effect was the shift in the +30 °C (B) band. This shift was observed even after 20 min of HCO_3^--depletion and continued until the peak shifted to 45 °C. The second effect was the reduction in the thermoluminescence intensity (of both A and B bands) which was evident only 2 h after HCO_3^- depletion (see Fig. 2B). The reduction in the thermoluminescence intensity of the B band is suggested to be due to a lowered incidence of $S_2Q_B^-$ recombination, but the reasons for the reduction of the A band needs to be further examined. The addition of HCO_3^- to a HCO_3^--depleted sample, even after 2 h of depletion, restored both the thermoluminescence intensity and the peak temperature of the B band. However, in these experiments, the A band was only partially restored. Obviously, there are some irreversible effects during the prolonged depletion procedure. Again, the −120 °C band was totally insensitive to HCO_3^- depletion confirming the data in Fig. 1.

The total intensity of thermoluminescence was affected only slightly by a mild HCO_3^- depletion (Fig. 1, curve 2; Fig. 2B); only after thylakoids had been depleted for 2 h by the continuous depletion method or by two washes in HCO_3^--depletion buffer did the thermoluminescence intensity decrease to a 50% level. However, this decrease in intensity could be fully restored upon the readdition of HCO_3^-. (This decrease in total intensity was also accompanied by a reduction in the −20 °C band.)

Light flash experiments

Since continuous illumination experiments are much more difficult to interpret than those with flash illumination, which can set the S states and the Q_B states into defined states, we performed experiments on the thermoluminescence of thylakoids after single saturating light flashes.

In mildly depleted thylakoids (depletion for 20 min, using the continuous depletion method), the thermoluminescence peak (B) after the first flash was shifted from 32 to 36 °C, with a slight increase in intensity (Fig. 3). The major reaction in flash 1 is the conversion of S_1Q_B to $S_2Q_B^-$ (see Eqn. 2). Thus, the shift is suggested to be due to the requirement of a higher activation energy [33] for $S_2Q_B^-$ recombination, although a change in frequency factor cannot be discounted; this may be most easily explained by a change in conformation of the Q_B-complex. The increased intensity of thermoluminescence, after the first flash, could be due to a decrease in the concentration of stable Q_B^- in dark-adapted HCO_3^--depleted thylakoids.

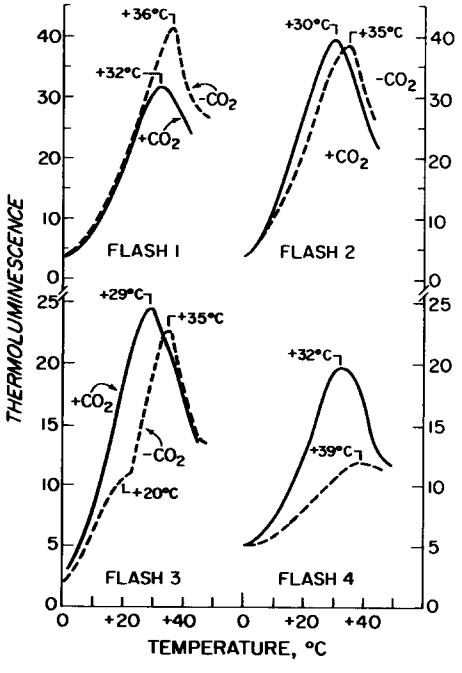

Fig. 3. Thermoluminescence as a function of temperature after four flashes of light in $CO_2(HCO_3^-)$-depleted (dashes) and reconstituted (solid line) spinach thylakoids. $CO_2(HCO_3^-)$-depletion was by the continuous depletion method for 20 min (mild-depletion). The dark time between flashes was approx. 1 s. Note that the ordinates are on slightly different scales, the left ordinates are for flashes 1 and 3, and the right for flashes 2 and 4.

If this is the case, a complementary decrease in $S_3Q_B^-$ recombination should be present after the second flash in HCO_3^--depleted thylakoids. A slight decrease was in fact observed (Fig. 3, flash 2). That the second flash generates a thermoluminescence band larger than the first is due to the presence of Q_B^- in the dark [24] and to the higher yield of S_3 luminescence in comparison with S_2 (Rutherford, A.W., Renger, G., Koike, H. and Inoue, Y., unpublished data; also see Ref. 38). However, HCO_3^--depletion did cause a shift from 30 to 35 °C. If $S_3Q_B^{2-}$ recombination exists, forming $S_2Q_B^-$, then the 35 °C (B) band may be due to the recombination of the latter. Flash 3 (note the change in scale), given 1 s after flash 2, also produced a shift from 30 to 35 °C; again, the intensity at 35 °C was slightly lower than at 30, but a shoulder appeared at 15–20 °C; this shoulder is probably due to recombination of some available $S_{2(3)}Q_A^-$. Flash 4 also shows the shift in the peak from 32 to 39 °C, but, here, we see a dramatic reduction in thermoluminescence intensity after HCO_3^--depletion. Untreated controls are similar to the HCO_3^--reconstituted samples (data not shown).

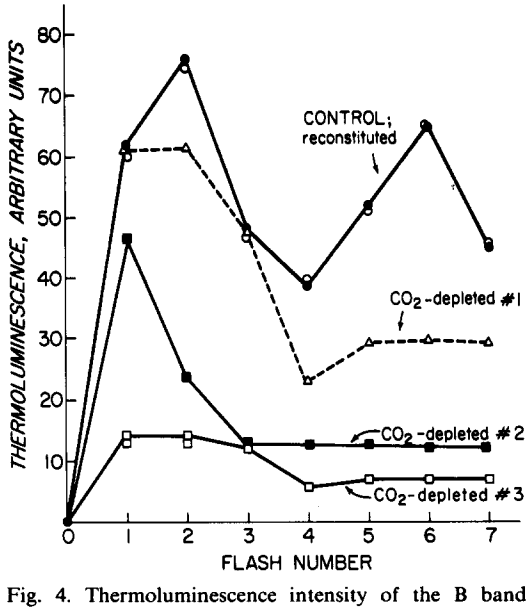

Fig. 4. Thermoluminescence intensity of the B band as a function of flash number for $CO_2(HCO_3^-)$-depleted, reconstituted and control spinach thylakoids. The dark time between flashes was approx. 1 s. CO_2-depleted number 1, sample depleted by the continuous depletion method for 30 min; CO_2-depleted number 2, sample depleted for approx. 1 h; CO_2-depleted number 3, sample depleted for approx. 3 h. The reconstituted sample (open circles) was for the sample depleted for 1.5 h to which 5 mM HCO_3^- was added; and the control sample (solid dots) was for a non-CO_2-depleted sample kept for 1.5 h.

It is not easy to quantitate these changes fully, because extensive HCO_3^--depletion may also cause a decrease in the efficiency of the back reaction. Fig. 4 shows our results with several different samples. Control and HCO_3^--reconstituted samples show an oscillation of thermoluminescence intensity of the B band: maxima are after flashes 2 and 6. Curves 1, 2 and 3 are for progressively greater depletion times. In most depleted (3 h) thylakoids, there was a significant decrease in the thermoluminescence intensity even after flash 1 (Fig. 4, CO_2-depleted curve 3), but the shift in emission temperature was clearly observed (as in Fig. 3). Even in the most mildly depleted (30 min) thylakoids (Fig. 4, CO_2-depleted curve 1), the thermoluminescence after flashes 4–7, which also showed the shift, had a decreased intensity. Since the samples in curve 1 (Fig. 4) had a milder depletion, and curve 3 (Fig. 4) had a more prolonged depletion than in Fig. 3, they cannot be quantitatively compared with each other. In all cases, however, the second cycle in the oscillatory pattern almost disappeared. In several experiments (curve 2, Fig. 4), the thermoluminescence intensity after flash 3 was as low as after flashes 4–7. This would be predicted from the following sequence of events, starting from dark-adapted state (also see Eqn. 2):

$$S_1Q_AQ_B \xrightarrow{1F} S_2Q_AQ_B^- \xrightarrow{2F} S_3Q_AQ_B^{2-} \xrightarrow{3F} S_0Q_A^-Q_B^{2-}$$

where F stands for flash. Here, recombination reactions between the electron acceptor side of PS II with S_0 is almost impossible and thus, there would be very low thermoluminescence intensity.

It has been suggested that the bicarbonate-depleted system might be stabilized in the $Q_A^-Q_B^{2-}$ state, since electron transfer is apparently (at least with dark times of approx. 30 ms) blocked after the third flash of a sequence [12]. If this were the only change, then the sequence of events, noted above, might be the only one expected to take place in the majority of PS II centers. In this scheme after three flashes, the electrons on the

acceptor side would be trapped on centers in which there were no acceptor side positive charges. Thus, thermoluminescence intensity would be low, as noted above, and would remain low on the third and subsequent flashes. This was not always observed (see below for further discussion). An interesting additional possibility is that bicarbonate depletion may inhibit the reassociation of the molecules of the plastoquinone pool with the Q_B-protein to produce the bound Q_B.

In darkness, the three other possible states (S_0Q_B, $S_0Q_B^-$ and $S_1Q_B^-$) are predicted, from the Kok model [5], to be in much lower concentrations. An electron-transfer block after Q_B should result in $S_{2(3)}Q_A^-(Q_B^{2-})$ recombination after the second and third flashes. Some indications of $S_{2(3)}Q_A^-$ recombination was indeed observed in HCO_3^- depleted thylakoids after three flashes (Fig. 3). It is evident, however, that the data cannot be described fully by this simple mechanism. In several experiments, thermoluminescence on the 4th flash was significantly smaller than after the 3rd flash (see Figs. 3 and 4) and no significant $S_2Q_A^-$ band could be detected after 2 flashes. The data might be better explained if other factors are taken into account which modify the simplified mechanism described above, e.g., (1) leakage of electrons out of the reaction center to the PQ pool; (2) the possibility of a high miss factor when in the $S_2Q_AQ_B^{2-}$ state due to the equilibrium: $Q_AQ_B^{2-} \rightleftarrows Q_A^-Q_B^-$; and (3) a progressive, but reversible, light-dependent inhibition of PS II might occur in the absence of HCO_3^- (or the presence of $HCOO^-$) with successive number of flashes. Such effects again could be explained by configurational (or conformational) change in the system resulting in increased non-recombination deactivation or increased non-luminescent recombinations.

It is important to emphasize that the addition of HCO_3^- to HCO_3^--depleted samples restores the flash pattern of the system to that observed in the controls (Fig. 4). Thus, the phenomenon is totally reversible. In spite of some variations in details, measurements on six HCO_3^--depleted samples showed the absence of the second cycle in the oscillatory pattern of thermoluminescence as a function of the flash number. In all cases examined, addition of HCO_3^- restored the thermoluminescence pattern to that of the controls (data not shown). However, further work using different conditions (e.g., either the temperature of illumination or dark times between flashes) might yield data to quantitatively explain all the results.

The oscillatory pattern of the control and reconstituted samples showed maxima at second and sixth flashes (Fig. 4). This pattern is different from that reported in Ref. 24 for completely relaxed thylakoids. The pattern observed here is partly due a higher efficiency of $S_3Q_B^-$ recombination (as noted above) and also partly due to a higher Q_B^-/Q_B ratio in the initial state here (see Discussion in Refs. 27 and 39). Possibly, the dim green safelight used in this study did not permit a complete relaxation in terms of Q_B^- although the S-state relaxation was complete. We note, however, that the possible incomplete relaxation of Q_B^- does not interfere with the conclusions derived from present experiments.

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