BICARBONATE: ITS ROLE IN PHOTOSYSTEM II

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Summary

The major role of the bicarbonate ion in isolated thylakoids is to accelerate the rate of the electron flow from the secondary electron acceptor R (or B) of the photosystem II to the plastoquinone pool (PQ). This conclusion is based on the measurements of chlorophyll *a* fluorescence after single flashes of light [5] and of absorbance changes of the plastoquinone system [18]. Independent biochemical measurements on the partial reactions confirm the conclusion that the major bicarbonate effect is located somewhere between the "primary" electron acceptor Q (or X-320) and the PQ pool [10]. The bottleneck reaction of the normal chloroplasts resupplied with bicarbonate is ≈20 ms (reoxidation of PQH_2). It is concluded that the bottleneck reaction of the bicarbonate-depleted chloroplasts is 100 to 200 ms, and this is located in the reoxidation of R^{2-}, thus explaining the 5-10 fold stimulation, by bicarbonate, of the steady-state saturation rates of the Hill reaction.

Introduction

In addition to its role as a substrate for carbohydrate production, CO_2 is required for the Hill reaction (see *e.g.* [21]). When chloroplasts are depleted of bicarbonate, the rate of the Hill reaction is inhibited; a large increase is, however, observed when bicarbonate is resup-

plied to such depleted samples. Earlier, Stemler and Govindjee [21-23] and Stemler *et al.* [20] believed that the absence of bicarbonate blocks the oxidizing (or water) side of pigment system II (PS II). Wydrzynski and Govindjee [30], on the basis of their data on chlorophyll *a* fluorescence transient, suggested a new site of bicarbonate action on the reducing side of PS II. Jursinic *et al.* [9] provided evidence for a major stimulatory effect of bicarbonate on the reoxidation of Q^- to Q as measured by the decay of chlorophyll *a* fluorescence yield after a saturating flash. Jursinic *et al.* [9] further concluded that there was no effect of bicarbonate on the electron flow from H_2O to the reaction center chlorophyll *a* of PS II (P 680).

In this paper we shall review recent experiments which show that the site of bicarbonate effect is between the "primary" electron acceptor Q and the plastoquinone (PQ) pool. A large stimulatory effect of bicarbonate on the reoxidation of the reduced secondary electron acceptor R^{2-} by the PQ pool appears to be the major effect of this anion in the thylakoid membranes.

A Few Characteristics of the CO_2 Effect

Effect of external pH on bicarbonate stimulation

The effect of bicarbonate as a function of external pH (5 to 9) shows a much larger stimulation in the Hill activity around pH 6 to 7, where the bicarbonate species predominates [10,21]. This could imply that the bicarbonate ion is the active species in stimulating the Hill reaction. However, the affinity of HCO_3^- to the membrane component(s) may be different at different pHs. No definite assertion can, therefore, be made regarding the active species involved.

Bicarbonate stimulation in the presence of uncouplers of photophosphorylation

Bicarbonate has also been shown to cause enhancement of photophosphorylation [1,16]. To distinguish between the direct stimulation of electron flow by HCO_3^- from any indirect stimulation produced by an enhancement of photophosphorylation by bicarbonate, Khanna *et al.* [10] measured the bicarbonate effect in the presence of several uncouplers of photophosphorylation. The data presented in Table 1 show that there are no changes in the rates of electron

TABLE 1

Bicarbonate effect in the presence of uncouplers of photophosphorylation: rates of O_2 evolution (after [10]).

	Electron transport, μequivalents/mg Chl per h		Ratio
	$-HCO_3^-$	+10 mM HCO_3^-	$+HCO_3^-/-HCO_3^-$
(1) Control	26 ± 3	171 ± 9	6.6
Plus NH_4Cl	24 ± 4	163 ± 7	6.8
(2) Control	22 ± 3	134 ± 14	6.1
Plus methylamine hydrochloride	23 ± 3	147 ± 18	6.4
(3) Control	22 ± 4	165 ± 15	7.5
Plus nigericin	21 ± 1	144 ± 5	6.9
(4) Control	13 ± 1	135 ± 4	10.4
Plus gramicidin-D	12 ± 1	125 ± 7	10.4

The 2 ml reaction mixture consisted of 50 mM phosphate buffer (pH 6.8), 100 mM sodium formate, 100 mM NaCl and 0.5 mM ferricyanide. Concentrations of uncouplers used were: NH_4Cl (1 mM), methylamine, HCl (0.3 mM), nigericin (5 μM) and gramicidin-D (1 μM). Spinach chloroplasts containing 33 μg chlorophyll (Chl) ml^{-1} suspensions were used. Average of three experiments is shown.

flow or in the bicarbonate stimulation in the presence of NH_4Cl, methylamine-HCl, nigericin or gramicidin-D. Our samples are uncoupled to begin with; this may be so because we use high concentration of salts in the depletion medium. In any case, these data clearly establish that the bicarbonate stimulation of Hill activity studied here is distinct from the effect of HCO_3^- on photophosphorylation.

However, we have not yet established if the HCO_3^- effect on photophosphorylation is through its effects on electron flow or through a direct effect on the coupling factor protein [13].

Decay of the Reduced Form of the "Primary" Electron Acceptor after Light Flashes

Bicarbonate effect on the chlorophyll a fluorescence yield

Measurement of the decay of chlorophyll *a* fluorescence yield, 50 μs to few ms after a light flash, suggests that the reoxidation of the reduced "primary" acceptor Q^- is inhibited by bicarbonate depletion [9]. The half time of decay of fluorescence is slowed down to about 3 ms in the CO_2-depleted sample. A five fold decrease in the half time of decay is observed when 10 mM bicarbonate is resupplied to the depleted sample (Fig.1). This data indicates that a bicarbonate action is on the dark reoxidation of Q^-

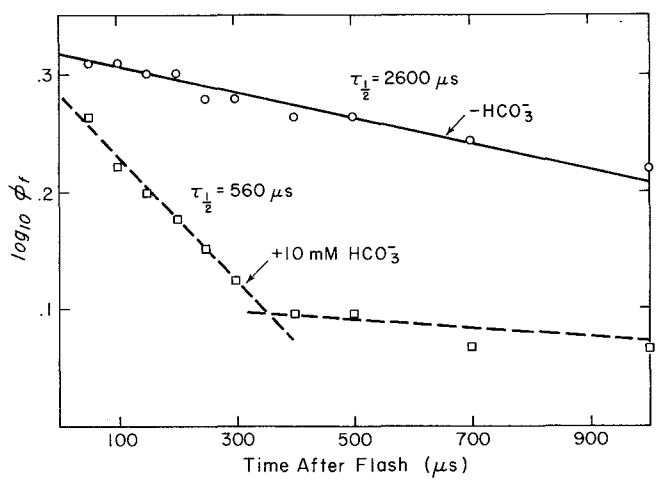


Fig. 1. Semilog. plot of the decay of chlorophyll *a* fluorescence yield (φf) after a saturing 10 ns 337 nm pulse with and without 10 mM bicarbonate. φf was measured with a variable delay analytic weak flash (General Electric Strobotac 1538-A; neutral density filters and Corning blue glass C.S. 4-96). Photomultiplier, EMI 9558 B protected with Wratten 2A and Schott RG-8 filters. *Lactuca sativa* chloroplasts were depleted of bicarbonate and resuspended in buffer as described by Wydrzynski and Govindjee [30]. Similar results were obtained with *Zea mays* chloroplasts (after [9]).

following a flash. Furthermore, the chlorophyll *a* fluorescence transients measured as a function of decreasing concentrations of bicarbonate are qualitatively similar to those observed with increasing concentrations of DCMU which imposes a block on the reducing side (see [30]). This also implies that bicarbonate depletion acts as a partial block of electron flow between Q and the intersystem carriers in much the same way as DCMU does at low concentrations.

Kinetics of absorption changes at 320 nm

Absorption changes at 320 nm indicate the formation and decay of semiquinone. Siggel *et al.*[18] studied the transition of quinone to semiquinone anion of the "primary" acceptor X-320 (Q) of PS II and its dark decay by monitoring absorption changes at 334 nm instead of at 320 nm (changes at 334 nm and 320 nm provided the same conclusions). Absorption change at 334 nm shows a very fast rise which is of the order of $\lesssim$ µs and a biphasic relaxation in dark with a half time of ∿500 µs for the faster component. The fast rise is attributed to the rapid photoreduction of X-320 and the 500 µs decay to the subsequent reoxidation of $X\text{-}320^-$. [$X320^-$ (or Q^-) donates its electron to the PQ pool *via* a two-electron acceptor species R (or B) [3,24].]

A semi-logarithmic plot of the time course of the absorption change at 334 nm, measured with repetitive flashes, shows two exponential phases of about equal magnitude with half-lifetimes ($t_{½}$) of 500 ± 100 µs and 7 ± 3ms in bicarbonate-depleted chloroplasts (Fig.2). The biphasic relaxation in these chloroplasts reflects heterogeneity of the sample introduced by the depletion procedure - about 30% of the total sample is unaffected by the procedure of depletion, another 30% exhibits slow kinetics; the rest of the signal (40%) was, however, totally inactivated, which is in agreement with the earlier conclusion

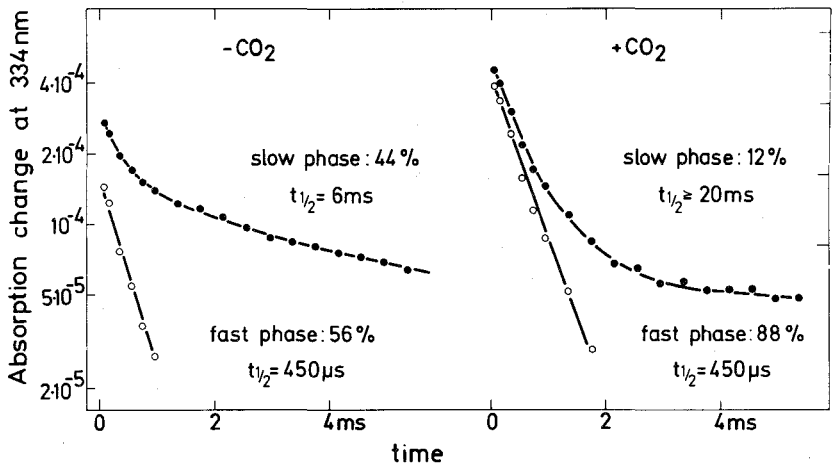


Fig. 2. Semilog. plots of the absorbance change at 334 nm induced by 20 µs repetitive flashes (indicating mainly Q^- and R^-, see text) for bicarbonate depleted ($-CO_2$) and reconstituted chloroplasts ($+CO_2$). *Spinacia oleracea* chloroplasts, which had been frozen and thawed, were depleted of bicarbonate as described earlier [21,30]. To obtain the $+CO_2$ sample, 20 mM bicarbonate was added. 512 flashes were averaged; the darktime between the flashes (td) = 250 ms; the electrical bandwidth was 10 kHz. The relative magnitude and half time of the two exponential phases are indicated (after [18]).

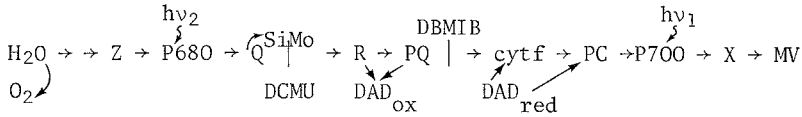
that about 30-40% of the reaction centre complex P680 is inactivated [9,20]. In the control and those chloroplasts reconstituted with bicarbonate, most of the signal decays with a $t_{\frac{1}{2}}$ of ∿500 µs, but, there is a minor proportion (10-15%) of the signal which decays with a half-lifetime of ∿20 ms. This is due to an uncharacterized signal probably due to the formation of plastohydroquinone or the reduction of oxidized P700.

Site of Bicarbonate Effect is Between Q and the PQ Pool

Partial biochemical reactions

To establish the site of the bicarbonate action by independent biochemical means, Khanna *et al.*[10] measured the electron flow in partial reactions. This was achieved by using appropriate electron donor-acceptor combinations in conjunction with specific inhibitors of electron car-

riers. The electron transport chain was divided as shown below:



where silicomolybdate (SiMo) accepts electrons from Q^- (see *e.g.*[31]), DCMU blocks electron flow from Q^- to R, oxidized diaminodurene (DAD_{ox}) accepts electrons somewhere before the plastoquinone pool [14], dibromothymoquinone (DBMIB) is an inhibitor of electron flow beyond the PQ pool [2], reduced diaminodurene (DAD_{red}) acts as an artificial electron donor to photosystem I [8], and methylviologen (MV) accepts electrons from X^-.

The bicarbonate effect in various partial reactions is shown in Table 2.

TABLE 2

Effect of Bicarbonate on Three Partial Reactions (after [10]*)*

System (See scheme in the text)	Electron transport μequivalents/mg Chl per h	
	$-HCO_3^-$	+10 mM HCO_3^-
(1) H_2O to silicomolybdate	117 ± 16	108 ± 17
(2) H_2O to oxidized diaminodurene	12 ± 1	90 ± 2
(3) Reduced diaminodurene to methyl viologen	662 ± 12	673 ± 16

Chloroplasts containing 33 μg Chl/ml were illuminated in a continuously stirred reaction mixture (2 ml) containing 50 mM phosphate buffer (pH 6.8), 100 mM sodium formate, 100 mM NaCl and the indicated donor and acceptor system. These systems were: (1) H_2O — silicomolybdate (SiMo); 5 μM 3-(3'4'dichlorophenyl)1,1'dimethylurea (DCMU) and 25 μM SiMo. (2) H_2O — oxidized diaminodurene (DAD_{ox}); 0.5 mM DAD, 0.5 mM ferricyanide and 0.5 μM 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). (3) DAD_{red} — methyl viologen (MV); 50 μM MV, 0.5 mM DAD, 2.0 mM sodium ascorbate and 1 μM DCMU. When SiMo or DAD_{ox} was the electron acceptor, electron transport was observed as O_2 evolution. When MV was the acceptor, electron transport was followed as O_2 uptake. All data have been converted to μequivalents of O_2 (or μmoles of electrons) mg (chlorophyll) $Chl^{-1}h^{-1}$. Average of three experiments is shown.

H_2O to silicomolybdate system shows no significant stimulation of O_2 evolution upon the addition of 10 mM HCO_3^- to the bicarbonate depleted samples. This implies that there is no major site of HCO_3^- action on the oxidizing side of photosystem II. The absence of bicarbonate causes a reversible inactivation of up to 40% of the photosystem II reaction centers (see refs [9,20]). Thus we had expected a small HCO_3^- effect on this reaction. However, this inactivation can range from 5 to 40% [12]. The absence of the HCO_3^- effect in the $H_2O \rightarrow$ SiMo reaction may have been due to the low inactivation of reaction center II in our preparations.

In the H_2O to oxidized diaminodurene reaction, a 7- to 8-fold enhancement of O_2 evolution is observed when 10 mM $NaHCO_3$ is added to the depleted samples. Oxidized diaminodurene, perhaps, intercepts electrons before they reach the last molecule in the PQ pool. The complete electron transfer chain from H_2O to X shows the same effect as the system just described. Since DBMIB prevents the electron flow out of the reduced PQ pool, we conclude that HCO_3^- acts somewhere between Q and the PQ pool.

The above conclusion is further confirmed by the observation that there is no effect of HCO_3^- depletion in the photosystem I electron transport chain ($DAD_{red} \rightarrow MV$).

Absorbance changes at 265 nm in repetitive flashes

Absorbance changes at 265 nm are indicative of changes in Q, R and the PQ pool. In the discussion below, the following flow diagram of the intersystem electron carriers will be used:

$$PSII \rightarrow Q \overset{(A)}{\rightarrow} R \overset{(B)}{\rightarrow} PQ \overset{(C)}{\rightarrow} \text{cyt f}$$

The dark relaxation of the absorption change at 265 nm has a major slow phase ($t_{½} \overset{\sim}{-} 20$ ms) (see *e.g.* [17]). The 20 ms time represents the oxidation of plastohydroquinone (reaction C). The control chloroplasts of this study behaved slightly differently than the normal chloroplasts

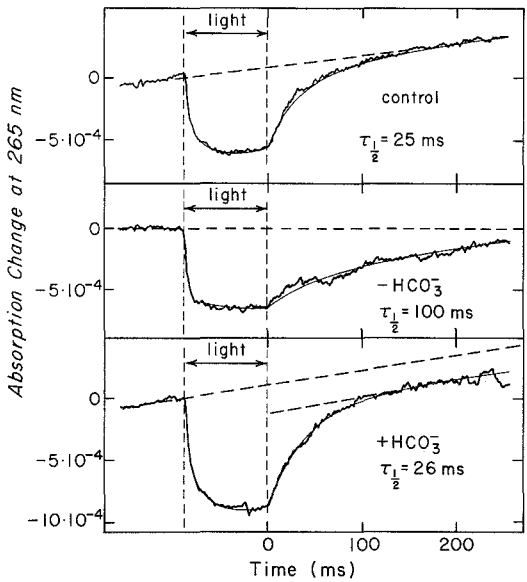


Fig. 3. Time course of the absorption change at 265 nm induced by 85 ms repetitive flashes (indicating mainly R^{--} and PQ^{--}, see text) for untreated (control), CO_2-depleted ($-CO_2$) and reconstituted ($+CO_2$) spinach chloroplasts (frozen and rethawed). The halftimes of dark relaxation are shown. Number of flashes, 64; darktime (td) = 5s; electrical bandwidth, 600 Hz. 720 nm background light ($\Delta\lambda$ = 15 nm) of 400 $erg.cm^{-2}.s^{-1}$ intensity was used except in the case of the control (leading to a reduced amplitude in this case; after [18]).

as the slow phase had a half time of 25 ms [18]. CO_2-depletion retards this slow phase from 25 ms to about 100 ms (Fig. 3). This effect is reversible as the addition of 10 or 20 mM bicarbonate restored the 25 ms phase. This slow phase could represent either (1) the oxidation of PQH_2, if the relaxation of the signal is governed by reaction C or, (2) reaction B, if the relaxation is determined by the formation of PQ^{--} from R^{--} (accompanied by no or a minor absorption change) and the absorption change is brought about by the consecutive faster oxidation of the PQH_2 (reaction C). We suggest that the first possibility holds for control and reconstituted chloroplasts and the second holds for bicarbonate-depleted chloroplasts; the latter is consistent with the experiments on

chlorophyll *a* fluorescence which show that a 150 ms time represents electron flow from R^{2-} to PQ pool in these depleted chloroplasts [5].

A retardation of the electron flow from R^{2-} to PQ explains the 5-10 times decreased rate of Hill reaction in the bicarbonate-depleted chloroplasts as the bottleneck reaction changes from a $t_{1/2}$ of $\sim$ 20 ms to 100-200 ms.

Bicarbonate effect on the secondary electron acceptor R (or B)

Govindjee *et al.*[5] studied the effect of bicarbonate depletion on the electron transport from the primary acceptor, Q, to the plastoquinone pool, *via* the secondary electron acceptor R (the two electron carrier). They measured the fluorescence yield 150 ms after the flash as a function of flash number. Fig. 4 shows that this fluorescence yield reaches a high level in the CO_2- depleted chloroplasts after the third and succeeding flashes. The fluorescence yields 150 ms after the 1st and 2nd flashes are, however, low. In control chloroplasts and depleted

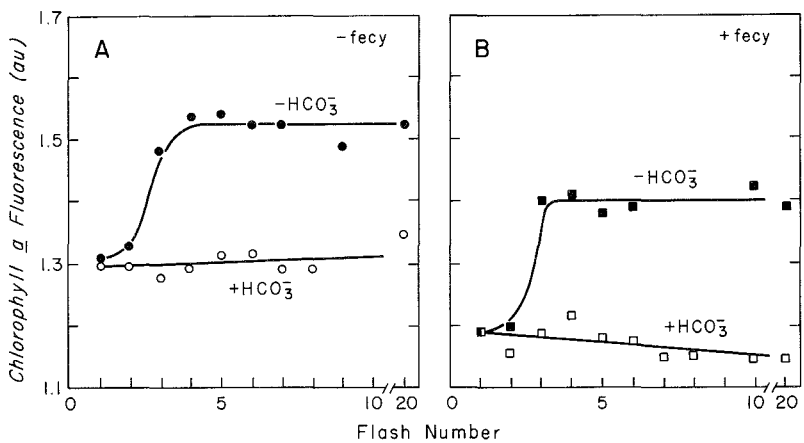


Fig. 4. Chlorophyll *a* fluorescence intensity ~150 ms after the last of a series of 3 μs saturating light flashes, spaced at 30 ms, as a function of the number of flashes. (au stands for arbitrary units.) Additions as indicated. Concentrations: Bicarbonate, 20 mM; chlorophyll, 20 $\mu g\text{-}ml^{-1}$ of spinach chloroplast suspension; ferricyanide (FeCy), 20 mM (after [5]).

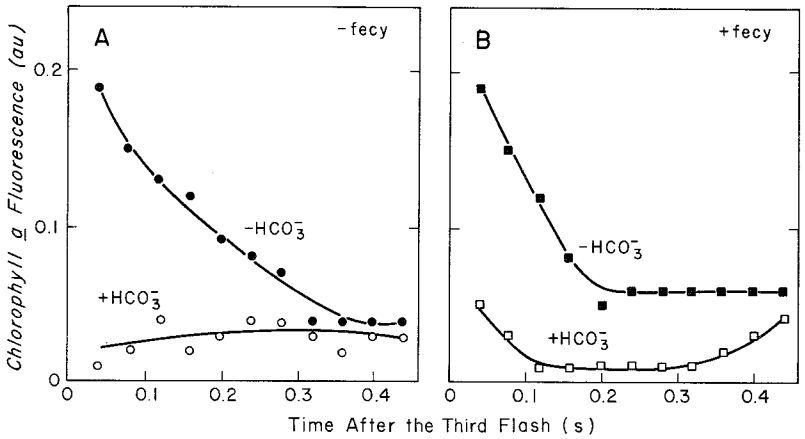


Fig. 5. Chlorophyll *a* fluorescence intensity after the third *minus* that after the second flash as a function of time. Additions as indicated; see the legend of Fig. 4 (after [5]).

chloroplasts resupplied with bicarbonate, the fluorescence yield is low after each of the light flashes. These results indicate that the fluorescence decay is slowed down from the third flash onwards in bicarbonate-depleted chloroplasts. The same results were obtained in the absence and the presence of ferricyanide. Thus, the observed effects were not influenced by the rate of electron acceptance by ferricyanide. Fig. 5 shows the kinetics of the slow fluorescence decay, plotted as the difference between the fluorescence intensity after the third minus that after the second flash, as a function of time. The half-time of this decay is of the order of 150 ms.

The above data are interpreted as follows. It is suggested that the absence of bicarbonate retards the electron flow from R^{2-} to the PQ pool. The first flash creates QR^{-}, the second flash QR^{2-}, and the third flash, if given fast enough, would form $Q^{-}R^{2-}$ and the fluorescence yield would be high. Succeeding flashes would give the same result. The 150 ms, noted above, would then reflect the $t_{½}$ of the reduction of PQ by R^{2-} in CO_2- depleted chloroplasts.

It thus appears, from the decay of chlorophyll *a* fluorescence yield after the third flash and of the decay of absorbance changes at 265 nm after repetitive long flashes, that the bicarbonate depletion slows down the rate of PQ reduction ($t_{\frac{1}{2}}$ of 100-200 ms); this becomes the new bottleneck reaction. In control and reconstituted chloroplasts, the bottleneck reaction has a $t_{\frac{1}{2}}$ of ∿20 ms (oxidation of PQH_2). Thus, the 5-10 fold inhibition of the electron flow in the absence of bicarbonate, or, else, the 5-10 stimulation of the Hill reaction by the addition of HCO_3^- to the depleted samples, can be finally explained.

Concluding Remarks

Warburg [27] believed that the requirement of CO_2 for the Hill reaction (which he had discovered [28]) was due to its involvement in the O_2 release in photosynthesis. This is the main rationale for the presentation of the present report at this symposium. The early work on the effect of CO_2 (or bicarbonate) on the Hill reaction was performed by several investigators (see refs [4,6,7,26,29]); Stemler and coworkers [19,24] and Metzner [12] have recently revived interest in this problem. Jursinic *et al.* [9] failed to find an effect of CO_2 on the oxygen evolution side of photosynthesis. The major effect of the bicarbonate ion has been shown here to be on the electron flow from the reduced form of the secondary electron acceptor (R^{2-}) to the plastoquinone (PQ) pool. The mechanism of this effect needs to be investigated. Perhaps, HCO_3^- is needed to place "R", which is a plastoquinone [15] in a proper conformation so that it can accept electrons from Q^- and donate electrons to the PQ pool in an efficient fashion. Kreutz [11] and Metzner [12] have suggested involvement of HCO_3^- in the O_2 evolution steps. However, all the recent experiments in our laboratory support a different site of HCO_3^- action. Thus, if there is any direct role of bicarbonate in the O_2 evolution, it still

needs to be discovered.

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