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Bicarbonate Ion as a Critical Factor in Photosynthetic Oxygen Evolution[1]

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ALAN STEMLER AND GOVINDJEE
Department of Botany, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Bicarbonate ion, not dissolved CO_2 gas, is shown to increase 4- to 5-fold the rate of dichlorophenol indophenol reduction by isolated maize (*Zea mays*) chloroplasts. Glutaraldehyde fixed chloroplasts continue to exhibit bicarbonate-dependent 2,6-dichlorophenol indophenol reduction. Bicarbonate is shown to act close to the oxygen-evolving site, *i.e.* prior to the electron donation site of diphenyl carbazide to photosystem II. Dark incubation and light pretreatment of chloroplasts in various concentrations of bicarbonate, just prior to assay, indicate that bicarbonate binds to chloroplasts in the dark and is released again as the Hill reaction proceeds in the light. It is suggested that bicarbonate ions may play a critical role in the oxygen-evolving process in photosynthesis.

The ability of CO_2 to stimulate the Hill reaction has been extensively studied but remains little understood. The discovery of this phenomenon was first claimed by Boyle (5), although his results were later questioned (1). The first unequivocal evidence was presented by Warburg and Krippahl (20; also see 19), and since then many workers have observed it (4, 6–8, 16, 17, 21). However, as was pointed out by Good (6), there is little agreement as to the conditions necessary for observing dependence of the Hill reaction on CO_2, let alone consensus on the significance of such dependence. It is the purpose of this paper to outline a method whereby large (4- to 5-fold) increases in the rate of ferricyanide or DCPIP[2] reduction by isolated chloroplasts can be observed consistently with the addition of bicarbonate ions and to present new data relevant to the site and mode of action of bicarbonate ions.

Experiments were done to determine whether bicarbonate ion or CO_2 gas is active in stimulating Hill reaction and whether glutaraldehyde-fixed chloroplasts would exhibit bicarbonate-dependent Hill reaction. Other experiments were done to attempt to locate more precisely the site of action of bicarbonate and to demonstrate the effects of dark incubation and light pretreatment of chloroplasts in various concentrations of bicarbonate.

MATERIALS AND METHODS

Maize (*Zea mays;* single cross hybrid GSC50) plants were grown in vermiculite under artificial light (using a combination of 60W tungsten and 40W fluorescent lamps; 12/12 hr, light/dark photoperiod) at room temperature and harvested 8–14 days after planting. Just prior to harvesting, the plants were placed in a closed chamber and illuminated with white light using a GE 100W flood lamp for 1 to 2 hr. Nitrogen gas was passed through the chamber during illumination to make it relatively CO_2-free. This procedure approximately doubled the bicarbonate effect observed subsequently with the isolated chloroplasts and made the observation of a large effect much more consistent.

After light treatment, the harvested leaves were cooled in ice water and ground in Serval Omni-Mixer (15 sec at 70 v, 10 sec at 100 v) at near freezing temperature using an isolation medium containing sucrose, 0.4 M; NaCl, 0.01 M; and NaH_2PO_4/Na_2HPO_4 buffer, 0.05 M, pH 6.8. The slurry was filtered through four layers of cheesecloth, and the filtrate was centrifuged for 1 min at 200*g*. The supernatant fluid was poured off and centrifuged for 10 min at 1500*g*. The resulting supernatant fluid was discarded. The chloroplasts comprising the pellet were then broken by suspension in sucrose-free buffer. The chloroplast suspension was centrifuged for 10 min at 1500*g*. The resulting pellet was resuspended in a small amount of isolation medium, divided into portions, and placed in a freezer. The frozen material was used usually within the following 2 weeks, during which time only a small loss of DCPIP-reducing ability occurred.

To deplete the chloroplasts of CO_2, *i.e.* bound bicarbonate, they were thawed and suspended in a solution containing 0.25 M NaCl, 0.04 M Na acetate, 0.05 M Na phosphate buffer, pH 5.0, which had been previously made CO_2-free by boiling or bubbling or both with N_2. The chlorophyll concentration was 50 μg/ml. A low pH was imperative for developing maximum bicarbonate dependence. With maize, only a small dependence developed if the chloroplasts were treated at a pH above 5.8. The optimum pH for this dependence was about 5.0, while below this activity was seriously impaired. Some experiments with oat chloroplasts showed a slightly higher optimum (about 5.4). The chloroplasts remained 30 min in the dark at 15 to 17 C while N_2 was bubbled through the suspension. After 30 min, aliquots were drawn off with a syringe and placed in cold screw-capped test tubes previously flushed with N_2. The material was centrifuged, the supernatant was poured off, and the tubes were placed in ice after once again being flushed with N_2. The bicarbonate-depleted chloroplasts were later resuspended in reaction mixture, and assays were conducted.

The most critical aspects of the bicarbonate-depletion procedure, noted above, are high anion concentration (chloride and acetate) to replace bound bicarbonate on the chloroplast and low pH to facilitate the conversion of bicarbonate to dissolve CO_2 gas.

Glutaraldehyde-fixed chloroplasts were prepared by the

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[2] Abbreviations: DCPIP: 2,6-dichlorophenol indophenol; DPC: diphenyl carbazide.

method of Park (15). To check fixation, glutaraldehyde-treated and untreated chloroplasts were subjected to osmotic shock and observed under the microscope. The unfixed chloroplasts were swollen, had poorly defined borders, were pale green in color, and showed no subchloroplast structure. Fixed chloroplasts were compact, had clearly defined edges, were dark green, and had a "grainy" appearance. The glutaraldehyde-fixed chloroplasts were then depleted of bicarbonate in the same manner as described above.

DCPIP reduction was measured using a Cary 14 recording spectrophotometer equipped with a side attachment to illuminate the sample. The actinic beam from a GE 120W, 650W, DVY lamp passed through a Corning C.S. 2-59 red cut-off filter, emerging with an intensity of 2×10^6 ergs cm^{-2} sec^{-1}. An appropriate interference filter (wavelength maximum, 597 nm; half band width, 12 nm) was placed in front of the photomultiplier to block out the actinic light so the change in absorbance of the sample, measured at 597 nm wavelength, could be monitored as it occurred. The reaction cuvette was flushed with N_2 and sealed, keeping the conditions initially anaerobic. Solutions were transferred from otherwise sealed vessels by means of syringes to minimize atmospheric contamination.

All reaction mixtures were heavily buffered and those with a high concentration of bicarbonate were brought back to the desired pH by the addition of dilute HCl. Solutions containing bicarbonate were kept sealed to prevent loss of CO_2 gas.

To prepare chloroplasts for the experiment in which the artificial electron donor DPC was used (18), they were placed in a water bath at 49 C for 5 min (10) immediately after treatment to deplete them of bicarbonate. This heat treatment destroyed the chloroplasts ability to reduce DCPIP without added electron donors except for trace amounts in the presence of bicarbonate. After heat treatment the chloroplasts were collected by centrifugation and used as usual.

The rate of DCPIP reduction was taken from the slope of the recorder trace starting with the onset of actinic illumination. These readings were converted into μmoles DCPIP reduced by using the extinction coefficient published by Armstrong (2) at pH 6.8, and relative coefficients were measured under our own conditions at other pH values. At very low bicarbonate concentrations, a definite lag period, lasting a variable number of seconds (1–15), was sometimes observed. When it occurred, the reaction rate was taken from the extent of the absorbance change at 1 min after the onset of illumination. Thus the lag does not play a significant role in the over-all effect discussed here. In the extreme case of a 15-sec lag in the bicarbonate-free system, the initial rate of DCPIP reduction, once it began, was still about a fourth of the rate seen at high concentrations of bicarbonate. To obtain consistent results, it was necessary to assay quickly after suspending the chloroplasts in reaction mixture.

Ferricyanide reduction was measured by the method of Avron and Shavit (3).

RESULTS

Active Form of CO_2 in Stimulating the Hill Reaction. Experiments were conducted to determine whether dissolved CO_2 gas or bicarbonate ion is the active species in stimulating Hill reaction. A bicarbonate concentration study was done at pH 5.8 and 6.8. If dissolved CO_2 were the active form, we would expect that a subsaturating concentration would show a larger stimulation of the Hill reaction at pH 5.8 than at pH 6.8. If bicarbonate were the active form, the same concentration should show a larger effect at pH 6.8 than at 5.8. Our results show that bicarbonate ions are the active species.

Table I. *Initial Rate of DCPIP Reduction as a Function of CO_2/HCO_3^- Concentration at pH 5.8 and 6.8*

The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer, 39 μM DCPIP, and 15 μg of chlorophyll/ml of chloroplast suspension. Saturating red light was 2×10^6 ergs cm^{-2} sec^{-1}. The data are the average of five experiments.

pH	$NaHCO_3$	Rate	$+HCO_3^-/-HCO_3^-$
	mM	*μmoles DCPIP reduced/mg Chl·hr*	*ratio*
5.8	0	17.3 ± 3.6	—
	0.1	17.6 ± 4.8	1
	0.5	23.3 ± 3.4	1.3
	1.0	24.4 ± 3.0	1.4
	5.0	38.2 ± 3.9	2.2
	20.0	78.8 ± 9.3	4.5
6.8	0	15.2 ± 2.6	—
	0.1	23.3 ± 5.1	1.5
	0.5	43.8 ± 6.5	2.4
	1.0	47.0 ± 4.9	3.1
	5.0	65.7 ± 2.7	4.3
	20.0	71.6 ± 5.1	4.7

The data presented in Table I show that at all CO_2/HCO_3^- concentrations below 20 mM, much larger stimulation in Hill activity is observed at pH 6.8 than at pH 5.8. For example, 0.5 mM $NaHCO_3$ increases the rate of Hill reaction 2.4-fold at pH 6.8 but only 1.3-fold at pH 5.8. At pH 6.8, 5 mM $NaHCO_3$ is nearly a saturating concentration producing a 4.3-fold increase, while this concentration, at pH 5.8, is still far below saturation, producing a 2.2-fold increase.

Similar results were observed when ferricyanide was used as a Hill oxidant. Again, low concentrations of bicarbonate stimulated Hill activity more at pH 6.8 than at pH 5.8.

Upon examining these data, one also notices a fairly abrupt 2-fold increase in the rate of DCPIP reduction in going from 5 to 20 mM $NaHCO_3^-$ at pH 5.8. We do not know the reason for this jump and hesitate to speculate extensively without establishing its true importance.

One may still argue that CO_2 is the active species by proposing that at the lower pH, the affinity of the active site for CO_2 is lowered so that even though CO_2 occurs at higher concentration at pH 5.8, it has less stimulating effect. We feel this is less likely, since it requires this added assumption. Moreover, it is difficult to imagine how unchanged CO_2 can possess the binding qualities required to explain other data presented later (results and discussion of Table IV).

Bicarbonate Stimulation of Hill Activity using Glutaraldehyde-fixed Chloroplasts. We have tested whether or not the bicarbonate caused some sort of gross conformational change in the chloroplast membrane, stimulating Hill reaction. It was reasoned that chloroplasts, in which such changes were blocked by glutaraldehyde fixation, would not demonstrate a bicarbonate effect. The effect was, however, present.

The results presented in Table II show that while glutaraldehyde treatment of chloroplasts reduces the effect of bicarbonate as compared to the normal (2.75-fold increase *versus* 4.78 for the normal), a large effect is still observed: The reduced bicarbonate effect may be attributed to nonspecific causes, since glutaraldehyde treatment also reduces the over-all activity (14). Similar results were obtained when ferricyanide was the Hill oxidant.

Bicarbonate Effect on an Artificial Electron Donor System. The following experiment was done to locate the site of action of bicarbonate along the electron transport chain. DPC, an

artificial electron donor to photosystem II (18), was given to chloroplasts made unable to evolve oxygen by heat treatment. It was expected that if bicarbonate acted between the oxygen-evolving site and the site of electron donation by DPC, no effect of bicarbonate would be observed on the rate of electron flow from DPC to DCPIP. This reaction was found to be insensitive to bicarbonate ions (Table III). Thus, at least one site of action of bicarbonate is before the site of donation of electrons by DPC, *i.e.* on the oxygen-evolving side of photosystem II.

Table II. *Initial Rate of DCPIP Reduction Using Normal and Glutaraldehyde-Fixed Chloroplasts in the Presence and Absence of Bicarbonate*

The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer, pH 6.8, 39 μM DCPIP, and 20 μg of chlorophyll/ml of chloroplast suspension. The saturating red light was 2×10^6 ergs cm^{-2} sec^{-1}. Initial condition was anaerobic. The data are the average of five experiments.

Chloroplasts	Rate		$+HCO_3^-/-HCO_3^-$
	$-NaHCO_3$	+0.02 M $NaCHO_3$	
	μmoles DCPIP/mg Chl·hr		*ratio*
Normal	16.0 ± 1.3	76.5 ± 6.6	4.78
Glutaraldehyde-fixed	8.25 ± 1.6	22.7 ± 2.4	2.75

Table III. *Initial Rate of DCPIP Reduction in Normal and Heat-treated Chloroplasts with DPC as Electron Donor with and Without Added Bicarbonate*

The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate 0.05 M phosphate buffer, pH 6.8, 39 μM DCPIP, and 15 μg of chlorophyll/ml of chloroplast suspension. Saturating red light was 2×10^6 ergs cm^{-2} sec^{-1}. The data are the average of five experiments.

Treatment	Rate		$+HCO_3^-/-HCO_3^-$
	$-NaHCO_3$	10 mM $NaHCO_3$	
	μmoles DCPIP reduced/mg Chl·hr		*ratio*
None	15.8 ± 3.8	70.0 ± 6.2	4.4
Heat	0.00	trace	
Heat + 0.05 mM DPC	50.0 ± 8.9	56.0 ± 11.6	1.12
Heat + 0.5 mM DPC + 50 μM DCMU	0.0	0.0	

Table III indicates that while normal chloroplasts show a large bicarbonate effect (4.4-fold increase with bicarbonate) when DCPIP reduction is coupled to the natural electron donor, only a very small effect is seen when DPC is the electron donor (1.12-fold increase with bicarbonate). Heated chloroplasts, without DPC, also show a trace more DCPIP reduction with bicarbonate. DCMU (50 μM) prevented DCPIP reduction when DPC donated electrons, as expected. The very slight stimulation of bicarbonate on the DPC/DCPIP system, if it is real, may be due to the stimulation of a trace amount of normal electron flow parallel to the artificial flow as seen when DPC is omitted from the reaction mixture.

Effect of Dark Incubation and Light Pretreatment at Various Bicarbonate Concentrations on Hill Activity. Table IV (lines 1 and 2) show that if normal chloroplasts, *i.e.* not HCO_3^- depleted, are incubated in 0.25 M NaCl and 40 mM Na acetate in the absence of bicarbonate, they tend slowly to lose activity. No loss is seen in the presence of 20 mM $NaHCO_3$. This confirms the findings of Good (6) and West and Hill (21). However, if one begins with bicarbonate-depleted chloroplasts, the reverse phenomenon can be observed. Comparing lines 3 and 4, it is apparent that, depending on bicarbonate concentration, a 5-min dark incubation period between suspending the chloroplasts in reaction mixture and assay can markedly increase the rate of reaction. In the absence of bicarbonate, and again at saturating concentrations (20 mM) a 5-min dark period has little effect. An incubation period of 5 min in 1 mM $NaHCO_3$, on the other hand, increases the initial rate of DCPIP reduction and, in 0.1 mM bicarbonate, the rate is doubled. In fact, incubating chloroplasts in the dark for 5 min at 0.1 mM $NaHCO_3$ produces the same initial rate of Hill reaction as 1 mM without dark incubation. The presence of electron acceptor (DCPIP) is not necessary during the incubation period (line 5).

Comparing lines 3 and 6, if instead of a dark period, Hill reaction is allowed to proceed in saturating light, no increase is seen in the rate of reaction after 2 min, regardless of the bicarbonate concentration. At high bicarbonate concentration (20 mM) an irreversible decrease is seen (a symptom of photo-

Table IV. *Initial Rate of DCPIP Reduction with Normal and Bicarbonate-depleted Chloroplasts as a Function of Bicarbonate Concentration and Various Light and Dark Pretreatments*

The reaction conditions were the same as in Table III. Chloroplasts were suspended in the reaction mixture containing 39 μM DCPIP for pretreatment except where indicated (rows 5 and 8), in which case the dye was injected immediately after pretreatment. The data are the average of five experiments.

Chloroplasts	Incubation Time and Pretreatment	Rate			
		$-HCO_3^-$	0.1 mM $NaHCO_3$	1 mM $NaHCO_3$	20 mM $NaHCO_3$
		μmoles DCPIP reduced/mg Chl·hr			
Normal	None	91.2 ± 4.7			85.5 ± 2.8
	5 Min dark	71.2 ± 7.8			82.0 ± 6.9
HCO_3^- depleted	None	15.2 ± 2.6	23.3 ± 5.1	47.0 ± 4.9	71.6 ± 5.1
	5 Min dark, + DCPIP	13.3 ± 3.7	48.5 ± 4.6	66.5 ± 8.3	75.0 ± 8.9
	5 Min dark, − DCPIP		47.3 ± 7.7		
	2 Min saturating light, + DCPIP	14.1 ± 2.2	24.7 ± 1.9	42.0 ± 1.6	59.4 ± 4.3
	2 Min saturating light, + DCPIP, + 5 min dark	18.3 ± 2.4	40.6 ± 2.7	64.3 ± 3.5	54.5 ± 6.7
	2 Min saturating light, − DCPIP, + 5 min dark				72.5 ± 6.3

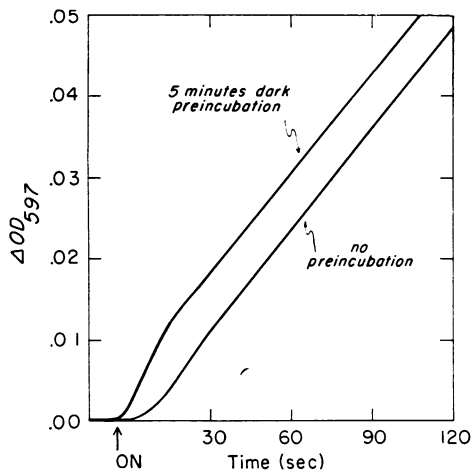

FIG. 1. Rate of DCPIP reduction by maize chloroplasts previously depleted of bicarbonate, with and without 5 min dark preincubation in 0.1 mM $NaHCO_3$. Other reaction conditions were the same as in Table III.

inactivation which is generally accelerated by increased electron flow; compare line 6 with line 8). Finally, if a 5-min dark period is given subsequent to 2 min of active Hill reaction, an increase in the initial rate is again observed at 0.1 mM and 1.0 mM bicarbonate (line 7).

Differences in the initial rate of DCPIP reduction with and without 5-min dark incubation are evident in the recorder traces presented in Figure 1. The bicarbonate concentration was 0.1 mM. When no dark time was allowed, the rate of reduction, after a short lag of a few seconds, is nearly linear for the first few minutes. With a 5-min dark preincubation, a high rate of DCPIP reduction upon illumination is first observed until a change in slope is seen between 10 and 20 sec. From that point onwards, the two curves run parallel, indicating nearly identical rates of reaction. The increased activity due to preincubation in the dark therefore is seen to be temporary. The curve for the 5-min dark incubated chloroplasts (Fig. 1) was selected to show the most rapid change in slope. Usually the change is more gradual, occurring over a number of seconds.

To explain these results, we propose that bicarbonate binds to active sites (reaction centers?) on the chloroplast in the dark. Moreover, it appears that bicarbonate may be released again from the chloroplast as the Hill reaction proceeds. We do not observe an increased rate with time if chloroplasts are suspended in bicarbonate and the Hill reaction is actually taking place (Table IV). The increased rate develops only in the dark. This suggests that while bicarbonate may be binding to the chloroplast even during the Hill reaction, it is released again at a corresponding rate. The kinetic differences in rate of DCPIP reduction seen in Figure 1 can also be explained in terms of a release of bicarbonate from the chloroplast. We propose that a reserve of (bound) bicarbonate is built up during 5-min dark incubation in chloroplasts suspended in 0.1 mM bicarbonate. This reserve accounts for the increased rate of reaction upon illumination. The change in slope occurs when this reserve is dissipated, *i.e.* bicarbonate is released. After the "excess" bicarbonate is released, the reaction rate is again dependent on the dark binding rate of bicarbonate. The proposal that bicarbonate is released during Hill reaction is further supported by other work in our laboratory showing that dependency of the reaction rate on bicarbonate increases as Hill reaction proceeds (manuscript in preparation).

DISCUSSION

Given the foregoing results, we may now explain some of the experimental inconsistencies related to the bicarbonate effect on the Hill reaction as well as answer some of the questions which have been raised.

The requirement of high salt concentration for maximum bicarbonate effect (6, 21) has not been fully explained. Since bicarbonate ion, not CO_2 gas, is shown to be the active moiety, it is reasonable to suggest that anions (chloride and particularly acetate) compete with bicarbonate for binding sites on the chloroplast. The higher the concentration of the ions, the more effective they will be at removing bicarbonate and keeping it from the active sites. Since the need for bicarbonate is very specific (6), such competitive action by other ions can effectively reduce activity. It is also of interest that once bicarbonate is removed from chloroplasts, bicarbonate dependence remains even after competing anions are removed. Further experiments in our laboratory (data not presented here) indicate that high salt concentrations are actually not necessary in the reaction mixture itself to see a large bicarbonate effect. As long as high salt concentrations are present during the bicarbonate depletion procedure, prior to assay, large bicarbonate stimulation can be observed in reaction mixture containing no acetate and only small amounts of NaCl. High salt concentration was left in the reaction mixture routinely only to minimize the effect of contamination of solutions with atmospheric CO_2.

West and Hill (21) pointed out that the bicarbonate concentration required to achieve saturation in their experiments differed from other workers, but offered no explanation. We now see that the effect of a given concentration of bicarbonate depends not only on the pH and the concentration of competing anions, but also on the time between suspending the chloroplasts in bicarbonate solution and assay. This time interval required for rebinding of bicarbonate is especially noticeable at lower concentrations and may very well have differed among investigators. The assumption of strong ionic binding characteristics of bicarbonate may also explain why certain investigators have seen little or no effect of exogenous bicarbonate on the Hill reaction (1). The bicarbonate already present must first be removed. In this regard, it is questionable whether or not CO_2 can be removed from alkaline solutions. The production of carbonate-free standard base solutions is not a simple matter (12). If most of the CO_2 is in the bicarbonate or carbonate ionic form, as is increasingly the case as one exceeds pH 7.0, it will not be removed by boiling or bubbling with another gas. Moreover, the presence of any organic matter to which these ions can bind may very well cause complications, making CO_2 removal even more difficult at high pH. Thus a failure to see an effect of exogenous bicarbonate at pH 8.0 (13), in contrast to a lower pH, can mean either that bicarbonate plays no role at that pH or, more likely, it was not adequately removed from the control.

To understand the bicarbonate effect, it is necessary to locate the site of action along the electron transport chain. Light intensity curves have previously been used to give a rough indication. If bicarbonate acts very near the early photochemical steps, we would expect to see some effect even at very low light intensity. Unfortunately, the published curves for DCPIP and ferricyanide reduction (21) are contradictory. It is therefore difficult to base firm conclusions on them. A failure to see a bicarbonate effect at low light intensity could also be explained by assuming incomplete removal of bound bicarbonate. While we have obtained our own light intensity curve (manuscript in preparation) showing the effect of bicarbonate on initial rates of ferricyanide reduction to be independent of

light intensity, we have also used more direct methods to locate the site of action of bicarbonate.

We note that electron donation by DPC to photosystem II via the primary oxidant Z is insensitive to bicarbonate (Table III). If we assume that there is an electron transport system between Z and the oxygen-evolving site, bicarbonate must act on that system. If, on the other hand, we assume that Z itself is part of the oxygen-evolving site, then we might postulate that, while DPC can donate electrons to the first oxidation state of the reaction center, *i.e.* Z^+, bicarbonate is somehow necessary for the accumulation of the four positive charges thought necessary for oxygen evolution, according to the present model of Kok *et al.* (11). In either case, bicarbonate must act very close to the oxygen-evolving site.

This conclusion is firmly supported by other work showing bicarbonate to have remarkable effects on both chlorophyll *a* fluorescence transients and delayed light emission characteristics of chloroplasts (manuscript in preparation). These parameters also indicate that bicarbonate is directly involved in the primary photochemical acts. Another site of action, related to phosphorylation, may also exist (4, 21).

Having more precisely located, at least, one site of action of bicarbonate, we must discuss the question of the role of this anion in the process of oxygen evolution. The discoverer of the CO_2 effect, Otto Warburg, cited this phenomenon as proof of his theory that oxygen is evolved as a result of splitting the CO_2 molecule and not from splitting H_2O. Those interested in the details of Warburg's radically different and now obscure model of oxygen evolution may consult reference 19. Perhaps now we can begin to understand Warburg's long insistence that CO_2 was necessary for oxygen evolution, even though few accept his general scheme of photosynthesis.

Not surprisingly, there are other possible modes by which bicarbonate can act besides that proposed by Warburg. (*a*) Originally, we worked under the hypothesis that bicarbonate may be an allosteric effector operating on the oxygen-evolving system. This may yet be the case, but the proposed strong binding characteristic would make bicarbonate a rather poor candidate for such control. (*b*) That bicarbonate causes large "configurational" changes can be ruled out by the glutaraldehyde experiment. Bicarbonate still may cause (micro) conformational changes in membrane protein, but this hypothesis is difficult to evaluate. (*c*) We may postulate that bicarbonate affects membrane potential. How this, in turn, would affect the primary photochemistry is not clear. Aside from the possible reversal of a cause-effect relationship, the question of specificity is raised. Good (6) has found that none of the many ions he tested substituted for bicarbonate. Still, it may be that only bicarbonate has this suggested effect on membrane potential. (*d*) It is also possible that bicarbonate is somehow stabilizing (or permitting a transition between) one or more of the oxidation states of Z which must accumulate 4 positive charges before oxygen can be released (11; also see ref. 9). This very interesting possibility, and the others mentioned, will be examined soon.

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