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BICARBONATE STIMULATION OF OXYGEN EVOLUTION IN CHLOROPLAST MEMBRANES

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INTRODUCTION

The ability of bicarbonate ion to stimulate (5-10 fold) Hill reaction in isolated chloroplast membrane fragments is now well documented (1-6), though very little is known about its precise role in the process. Bicarbonate ion, rather than CO_2, appears to be the active species (7). A more extensive investigation (7-9) of this phenomenon has yielded surprising results and indicates that HCO_3^- has a more critical role in oxygen evolution than previously suspected. Our recent experiments and conclusions (7-9) will be summarized below.

INCREASE IN HCO_3 DEPENDENCE WITH PREILLUMINATION

Isolated oat (Avena sativa) chloroplast membranes (see ref. 7 for details), not previously depleted of HCO_3^-, were placed on the surface of a platinum rate electrode that was polarized at +0.7 volt relative to the Ag/AgCl electrode so as to measure ferricyanide production (see ref. 9 for details). The chloroplast preparation (to be referred to as chloroplasts for brevity) was preilluminated with saturating white light in the presence of ferricyanide for a variable length of time while HCO_3^- free solution passed over the membrane holding the chloroplasts to the surface of the platinum (see legend of Fig. 1 for the composition of the suspension medium). After this period

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Abbreviations: DCPIP - 2,6-dichlorophenol indophenol; DPC - diphenyl carbazide; DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

of illumination several half-second assays were conducted. The chloroplasts were then provided HCO_3^- containing solution and several more half-second assays were performed.

The ability of preillumination to enhance dependence of the Hill reaction on HCO_3^- is shown in Fig. 1.

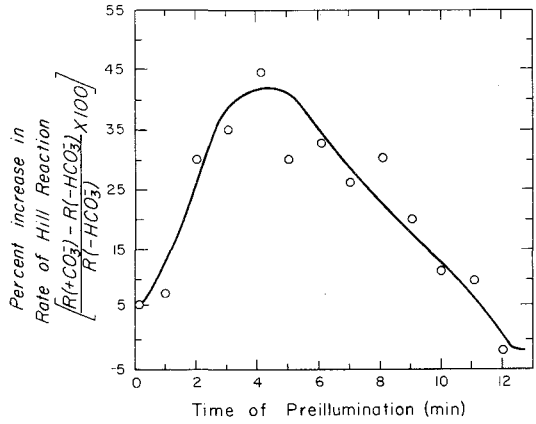

Fig. 1. Percent increase in the rate of ferricyanide reduction with added HCO_3^- as a function of preillumination time. The solution passing over the electrode membrane contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate pH 6.8, 0.5 mM potassium ferricyanide ±0.01 M $NaHCO_3$. Saturating white light (10^3 W/m^2) used during preillumination and half-second assays. Anaerobic conditions. Oat chloroplast membrane fragments (43 µg chlorophyll/ml in stock). Average of two series [after Stemler and Govindjee (9)].

Progressive increase in HCO_3^- dependence continues for the first four minutes of preillumination. Dark controls, and chloroplasts preilluminated in the absence of ferricyanide, showed no change in dependence on HCO_3^-. It should be mentioned that the decline in HCO_3^- dependence after 4 minutes is associated with a decline in the overall activity, i.e., it reflects photoinactivation.

Since $H^{14}CO_3^-$ was not incorporated (A. Stemler, unpublished) into a stable compound during Hill reaction, and thus "used up", increased dependence on HCO_3^- with time of illumination suggests that this ion is initially bound, perhaps ionicly to reaction centers directly, or exists in some complexed condition. As Hill reaction proceeds, it may become unbound or otherwise free (possibly as CO_2?).

BICARBONATE EFFECT AS A FUNCTION OF LIGHT INTENSITY

Oat chloroplast fragments, again not previously depleted of HCO_3^-, were placed on the platinum rate electrode and allowed to perform Hill reaction in the

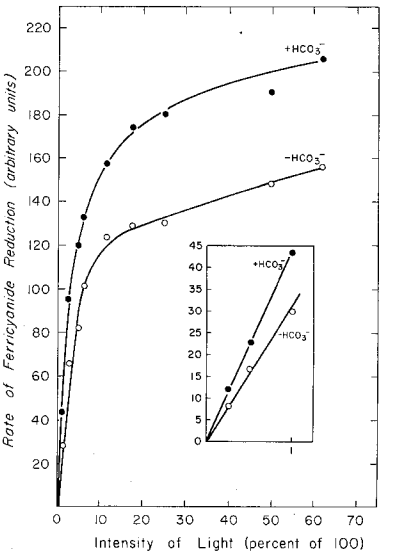

Fig. 2. Rate of ferricyanide reduction with and without 0.01 M $NaHCO_3$ as a function of light intensity. Oat chloroplast fragments preilluminated 4 min. in saturating white light. Ligh intensity was varied with calibrated neutral density filters. Average of 3 series. Other conditions as in Fig. 1 [after Stemler and Govindjee (9)].

absence of exogenous HCO_3^- for 4 minutes to induce a degree of dependence on HCO_3^- as shown in Fig. 1. Half-second assays were then conducted without, and then with, HCO_3^- as described above. In this case light intensity was varied by means of calibrated neutral density filters. Under these conditions the bicarbonate effect is independent of light intensity as shown in Fig. 2. Even at the lowest intensity where ferricyanide reduction could be accurately measured (see insert), HCO_3^- stimulated the activity to the same degree as at saturating intensities, or about 35 percent at all intensities. We conclude from the results presented in Fig. 2 that HCO_3^- is involved in early photochemical reactions of photosystem II rather than purely enzymatic reactions somewhat removed from the reaction centers.

COMPARISON OF THE EFFECT OF HCO_3^- ON OXYGEN EVOLUTION AND FERRICYANIDE REDUCTION MEASURED SIMULTANEOUSLY

Maize (Zea mays) was grown and chloroplast membrane fragments isolated from them as described earlier (7). Chloroplasts, to be depleted of HCO_3^-, were suspended in a solution containing 0.25 M NaCl, 0.04 M Na acetate, and 0.05 M Na phosphate, at pH 5.0, (see reference 7 for further details). The chloroplasts remained in this solution for 30 minutes at room temperature while N_2 gas was bubbled through the suspension to remove CO_2. Both high salt content and low pH were necessary for developing maximum HCO_3^- dependence. Ferricyanide reduction and oxygen evolution were measured as described in ref. 9.

When maize chloroplast fragments were depleted of HCO_3^-, over 90% of their oxygen evolving ability was suppressed while at the same time their ability to reduce ferricyanide was suppressed less than 80%. This is shown in Fig. 3. If HCO_3^- was present, however, equal μ equivalents of oxygen and ferricyanide were produced, at least during the first several minutes of illumination. That is, in the presence of HCO_3^-, for every molecule of oxygen evolved, four electrons are transferred to ferricyanide.

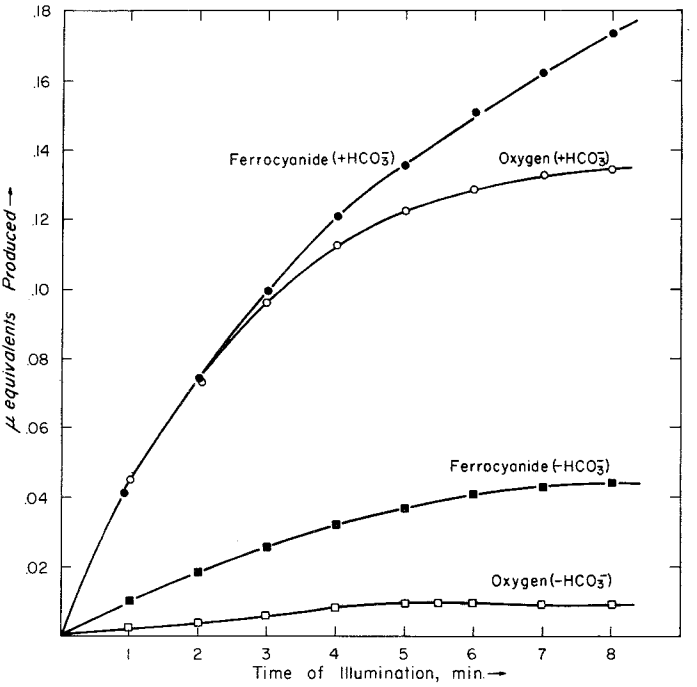

Fig. 3. Comparison of oxygen evolution and ferricyanide reduction in the presence and absence of HCO_3^-. Reaction mixtures contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate, pH 6.8, 1 mM potassium ferricyanide ±0.01 M $NaHCO_3$ and 15 μg chlorophyll/ml of maize chloroplast membrane fragments suspension. The light intensity was 500 W/m^2. Corning C.S. 3-71 (yellow) cut-off filters were used [after Stemler and Govindjee (9)].

These results can be explained by assuming the presence of a fairly substantial amount of some endogenous electron donor capable of reducing ferricyanide without evolving oxygen (see section 4 below). This assumption is consistent with the data of Kahn (10) and more recently of Huzisige and Yamamoto (11) who showed residual ferricyanide reduction in the absence of oxygen evolution in chloroplast particles. However

TABLE I
RATE OF DCPIP REDUCTION WITH
AND WITHOUT BICARBONATE

Treatment	Rate		$+HCO_3^-$ / $-HCO_3^-$
	$-NaHCO_3$	$+0.01$ M $NaHCO_3$	
	µmoles DCPIP reduced/mg Chl/hr		Ratio
None	15.8 ± 3.8	70.0 ± 6.2	4.4
Heat	0.00	Trace	--
Heat + 0.05mM DPC	50.0 ± 8.9	56.0 ± 11.6	1.12
Heat + 0.5mM DPC + 50 µM DCMU	0.0	0.0	--

Initial rate of DCPIP reduction in normal and heat treated (maize) chloroplast fragments 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 chlorophyll/ml. Saturating red light was 2×10^3 W/m^2. The data are the average of five experiments [after Stemler and Govindjee (7)].

the identity of this endogenous donor, or its possible role in "normal" electron flow, is not known. In any case, HCO_3^- appears to direct electron flow away from this donor and "couples" it rather to oxygen evolution.

EFFECT OF BICARBONATE ON AN ARTIFICIAL ELECTRON DONOR SYSTEM

To test whether HCO_3^- acts near the oxygen evolving site or farther along the electron transport chain, an artificial electron donor to photosystem II, diphenyl carbazide (DPC), was given to maize chloroplast membranes made unable to evolve oxygen by heat treatment. It was reasoned that if HCO_3^- 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 the electron acceptor (the Hill oxidant). The acceptor chosen for the present study was DCPIP, measured spectrophotometrically as described in ref.7.

Table 1 indicates that while normal (unheated) chloroplast fragments show a large HCO_3^- effect (4.4 fold increase with HCO_3^-) when DCPIP reduction is coupled to the natural electron donor, no significant effect is seen when DPC is the electron donor (1.12-fold increase with HCO_3^-).

We conclude from the data in Table I that at least one site of action of HCO_3^- is at, or very near, the oxygen evolving mechanism.

THE EFFECT OF BICARBONATE ON CHLOROPHYLL *a* FLUORESCENCE TRANSIENTS

Chlorophyll *a* fluorescence transients were measured as described in ref. 8. Figure 4 shows the fast fluorescence transient observed in isolated maize chloroplast fragments exposed to $80 W/m^2$ blue-green light. While constant fluorescence (F_0 or "0" level) is not affected in HCO_3^- depleted chloroplasts (see ref. 8), the initial fluorescence rise from the first recorded level to "I" is rapid, but the I → D

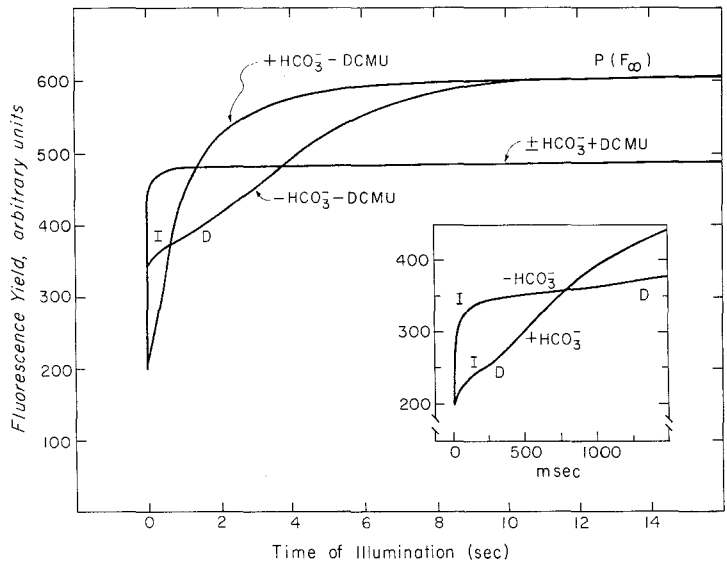

Fig. 4. Fluorescence yield of chlorophyll a at 685 nm as a function of time of illumination in the presence and absence of 0.01 M $NaHCO_3$. Maize chloroplast fragments previously depleted of HCO_3^- were suspended in 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer pH 6.8, ±10 μM DCMU; 10 μg chlorophyll/ml. Blue actinic light, 80 W/m^2. [After Stemler and Govindjee (8).]

(the inflection) and the D → P (peak F_∞) phases are slow. In the presence of 10 mM HCO_3^-, the initial fast rise to I appears slower and is depressed, the level D is not clear and occurs earlier (see insert Fig. 4), and the D → P rise is much more rapid with a half rise time of about 1 vs 4s in bicarbonate-depleted chloroplasts. The P level fluorescence, like that of the 0 level, is insensitive to HCO_3^-.

To explain Chl a fluorescence transients, it is generally assumed (see Duysens and Sweers, 12) that the yield of variable fluorescence reflects the redox state of Q, the primary electron acceptor of photosystem II. HCO_3^- depletion may block electron flow either before or after Q. However, if the block occurs

after Q, preventing the reoxidation of Q by intersystem intermediates (A pool), variable fluorescence should be at all times greater in the absence of HCO_3^-. Since this is not the case, we feel that the effect of HCO_3^- on fluorescence transients provides further evidence that HCO_3^- is acting on the oxygen evolving side of photosystem II (for further analysis, see ref. 8).

THE EFFECT OF BICARBONATE ON DELAYED LIGHT EMISSION

Delayed light emission (DLE) measurements were made as described in ref. 8. Maize chloroplast fragments were illuminated for 60 sec with low intensity (0.4 W/m^2) blue light. When observed in the time period starting about 0.5 sec after the cessation of illumination, the greatest amount of DLE results when HCO_3^- was resupplied during illumination (see Fig. 5.) Relatively less (about 70% of the maximum) DLE is observed when DCMU is also present; the omission of HCO_3^- causes an even greater decline in DLE to about 50% of the maximum. If HCO_3^- is omitted and DCMU is present, DLE is almost completely quenched, only about 5% of the maximum remaining.

Since DLE, in the time period observed here, is usually considered to be due to back reactions (i.e. charge recombinations) in photosystem II reaction centers the effect of HCO_3^- appears puzzling. HCO_3^- is known to stimulate oxygen evolution (1-4) and ferricyanide and DCPIP reduction (5-7) indicating greater photochemical efficiency of photosystem II. At the same time back reactions leading to increased DLE are also stimulated, arguing for less overall efficiency. To render these observations compatible, we refer to the kinetic model of oxygen evolution of Forbush et al. (13) and propose that HCO_3^- permits the formation of higher oxidation states ($>S_1$) of the photosystem II reaction centers. Such states are not only necessary for oxygen evolution, but are also the only states with significant propensity to decay (back react) yielding much more DLE (14, 15) (for more detailed discussion of HCO_3^- effects on DLE, see ref. 8.)

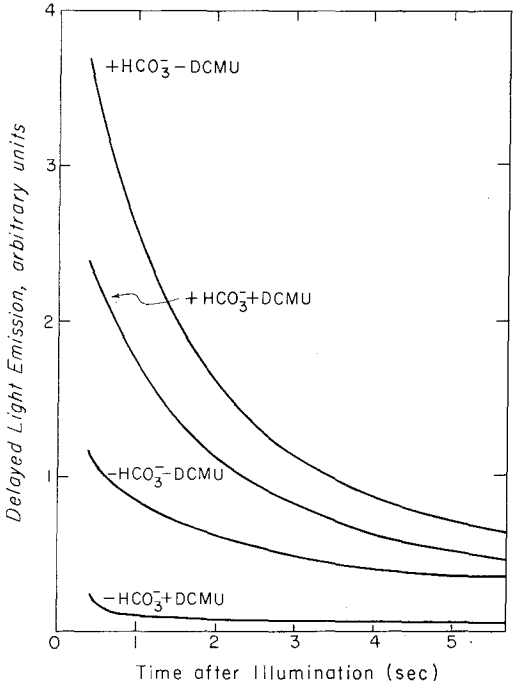

Fig. 5. Delayed light emission decay from maize chloroplast fragments, previously depleted of HCO_3^-, illuminated for 60 s. in weak blue light (0.4 W/m^2). Chloroplast fragments were suspended in 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate buffer pH 6.8, ±0.01 M $NaHCO_3$ ± 10 µM DCMU; 15 µg chlorophyll/ml. [After Stemler and Govindjee (8).]

CONCLUSIONS

From the evidence presented here and elsewhere we conclude that HCO_3^- has some important role in the oxygen evolving process. One possible mode of action has been eliminated. HCO_3^- does not in any way change the spillover of excitation energy between photosystem II and photosystem I as inferred from the absence of any effect of HCO_3^- on the fluorescence emission spectra both at room and liquid nitrogen temperatures (T. Wydrzynski and Govindjee, unpublished.)

Many other possible mechanisms of action remain. Otto Warburg (1) 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. There are, of course, other possible modes of action. Metzner (16) has published a scheme suggesting that the bicarbonate ion, bound to an acceptor, may function as the electron donor of photosystem II. HCO_3^- may be simply an allosteric effector operating on the oxygen evolving enzyme. It may be causing conformational changes in membrane protein or influencing membrane potential in some way. We now have some evidence (Stemler et al., in preparation (17)) that HCO_3^- is, in some manner, facilitating the formation of higher oxidation states of the photosystem II reaction centers. Since none of the above possibilities have been completely ruled out we are continuing the investigation of this phenomenon.

SUMMARY

Dependence of ferricyanide reduction (Hill reaction) by broken chloroplast membranes on bicarbonate ion increases with time of illumination. The stimulation caused by HCO_3^- is clearly observed even at low light intensities, suggesting an involvement of this anion in early photochemical reactions. The greater dependence of oxygen evolution than of ferricyanide reduction on HCO_3^-, as reported here, is interpreted to indicate the presence of an endogenous, non-oxygen evolving electron donor. Electron flow from the artificial electron donor diphenyl carbazide (DPC) to dichlorophenol indophenol (DCPIP) through photosystem II, in contrast to normal flow, is insensitive to HCO_3^-, indicating that HCO_3^- acts at or near the oxygen evolving site. Effects of HCO_3^- on chlorophyll *a* fluorescence and delayed light emission also suggest that this ion acts on the oxygen evolving side of photosystem II. We conclude that HCO_3^- has an important and a critical role in the oxygen evolution steps of photosynthesis.

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