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Absence of a Formate-Induced Release of Bicarbonate from Photosystem II[1]

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ABSTRACT

Formate has been proposed to inhibit electron flow in photosystem II by replacing endogenous bound bicarbonate on the reaction center complex. A mass spectrometer was used to measure directly the CO_2/HCO_3^- released when maize thylakoids, showing normal rates of electron flow, were treated with formate. Although the formate inhibited electron flow by 95%, no release (displacement) of CO_2/HCO_3^- was detected. This is consistent with the concept that membrane-bound HCO_3^- is not a requirement for normal rates of electron flow through photosystem II. Moreover, formate and other monovalent anions do not inhibit electron flow by removing bound HCO_3^- but by binding to empty sites. The "bicarbonate effect" is a reversal, by high concentrations of exogenous bicarbonate, of anion inhibition of photosystem II.

High concentrations of monovalent anions (formate, acetate, nitrite, etc.) inhibit electron flow through photosystem II (5, 21, 23). There is disagreement as to how this comes about. Because the inhibition is reversed by high concentrations of bicarbonate, it has been assumed that inhibitory anions acted by removing endogenous bound HCO_3^- from the reaction center complex. (Experiments done to date indicate that HCO_3^-, not CO_2, is active [1]. Since in the present work CO_2 was the species actually measured, the term CO_2/HCO_3^- will be used where appropriate.) It has been further suggested that bound HCO_3^- is either a requirement for photosystem II electron transport or an important regulator. (For reviews assuming this mode of action, see refs. 6, 17, 24, 25.) More recently, this interpretation has been questioned (9, 13, 19), based on the high binding constant for HCO_3^- of 80 to 100 μM CO_2/HCO_3^- at pH 6.5. Chloroplast thylakoids suspended in HCO_3^--free medium at pH 6.5 should have no bound HCO_3^-, yet electron flow proceeds normally unless high concentrations of inhibitory anions are present. This implies that anions inhibit electron flow by binding to *empty* sites on photosystem II. In this view, bicarbonate, because of its unique properties (for discussion, see ref. 18), does not inhibit electron flow and thus can reverse the effects of other anions when it replaces them on the photosystem II complex. The implication in this case is that endogenous bound HCO_3^- is not a requirement for photosystem II activity. This second interpretation has not been accepted (3, 22) if one can judge by the continued use of the term "HCO_3^--depleted" chloroplasts to mean "formate-pretreated" or "anion-inhibited" thylakoids. To test which of these views is correct, a mass spectrometer was used to measure the amount of CO_2/HCO_3^- given off, or displaced, when maize thylakoids, showing normal rates of electron transport, were treated with formate. It was found that, under conditions in which added formate produced a 95% reduction in electron flow rate, no CO_2/HCO_3^- was released from the thylakoids. This result is consistent with the hypothesis that formate binds to empty sites.

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MATERIALS AND METHODS

Maize (*Zea mays*) plants were grown in a greenhouse, and broken chloroplasts were isolated as previously described (16). The grinding medium contained 0.05 M sodium phosphate (pH 7.0), 0.4 M sucrose, 5 mM $MgCl_2$. After the final centrifugation, the pellet was resuspended in a small amount of grinding medium and frozen at −80°C. Just before the experiments began, the thylakoids were thawed and washed once in a fivefold excess of reaction mixture that contained 0.05 M sodium phosphate (pH 6.0) and 0.05 M NaCl, and then collected by centrifugation. The pellet was resuspended in a small amount of reaction mixture, kept on ice, and used in either the Hill reaction measurements or mass spectrometry experiments.

Oxygen evolution supported by added electron acceptors was monitored with a Rank-Brothers, Clark-type electrode system. The light source was a Sylvania 500-W projector lamp, and the light was focused with a slide projector. Samples were illuminated with saturating white light of approximately 500 W/m^2. The temperature was 25°C.

CO_2 in the chloroplast samples was measured with a VG Trio-2 mass spectrometer. A Plexiglas reaction chamber was separated from the vacuum line leading to the instrument by means of a General Electric MEM-213 membrane. The sample was stirred constantly during the measurements with a magnetic flea. The temperature was kept constant at 25°C.

RESULTS

Control chloroplasts illuminated at pH 6.0 in the presence of electron acceptors showed normal rates of oxygen evolution (Table I). Chloroplasts given 10 mM $NaHCO^-{}_3$ in addition showed essentially the same rate as the controls. In view of the alternatives discussed in the Introduction, this means that either the controls already had a full complement of membrane-bound HCO_3^-, or that photosystem II was operating normally with empty binding sites. When formate was in-

Table I. *Hill Reaction Rates in Maize Thylakoids Treated with Either Bicarbonate or Formate*

The reaction mixture contained 0.05 M sodium phosphate, pH 6.0, 0.05 M NaCl, and 20 μg of Chl mL^{-1}. After baseline was achieved in the dark, either H_2O (control), $NaHCO_3^-$ to 10 mM, or sodium formate to 100 mM was injected. One minute after injection, light was applied. Just before illumination, the following additions were made: gramicidin to 3 μM, 2,6-dichloro-*p*-benzoquinone to 200 μM, and $K_3Fe(CN)_6$ to 1 mM. The temperature was 26°C. Initial rates are expressed. The standard error was calculated from five measurements under each condition.

O_2 Evolved		
Control	+10 mM CO_2/HCO_3^-	+100 mM Sodium formate
	μmol mg^{-1} Chl h^{-1}	
377 ± 41	346 ± 32	23 ± 2.5

jected into chloroplast suspensions to a final concentration of 100 mM, and light was given 1 min later, the rate of oxygen evolution was reduced by 95%. This means that, in less than 1 min, formate had either removed nearly all of the bound HCO_3^- or had bound to empty sites.

A more cumbersome variation of this experiment was done in which chloroplasts were treated with formate at much higher Chl concentrations. Such concentrations (5 mg of Chl mL^{-1}) were required for the mass spectrometer experiments to be described later. Thylakoids were washed once and resuspended in a small amount of the reaction mixture described in the Table I legend. Final Chl concentration was 5 mg mL^{-1}. For the controls, aliquots of this stock suspension were injected into an already illuminated reaction vessel that contained reaction mixture including uncoupler and Hill oxidants. Oxygen evolution was measured immediately. Experimental samples were drawn from similar chloroplast stock suspensions that were also given 100 mM sodium formate. Aliquots were injected into the illuminated reaction mixture 1 min after formate addition to the chloroplast stock suspension. The reaction mixture was identical to that of the controls, except for the presence of 100 mM sodium formate. The results of these experiments (data omitted) were essentially the same as for the simpler protocol described in the preceding paragraph and Table I. This indicates that formate inhibition is independent of chlorophyll concentration over a wide range. This is expected in theory whenever binding affinity is weak. In this case, the ratio of ligand (100 mM formate) to binding site (approximately 10 μM photosystem II at 5 mg of Chl mL^{-1}) is so great that chloroplasts cannot be concentrated enough to have more than a negligible effect on the free ligand concentration.

To decide whether formate displaces bicarbonate or binds to empty sites, similar experiments were done in a reaction chamber attached to a mass spectrometer. Dissolved CO_2 in the chamber can be measured as mass 44. In control measurements (Fig. 1A) the reaction chamber was filled with reaction medium alone; no thylakoids were present. After several minutes, data acquisition was begun and the spectrometer recorded mass 44 (CO_2). At 3.0 min, sodium formate was injected to a concentration of 100 mM. This caused a slight inflection upward, probably due to a formate-induced change

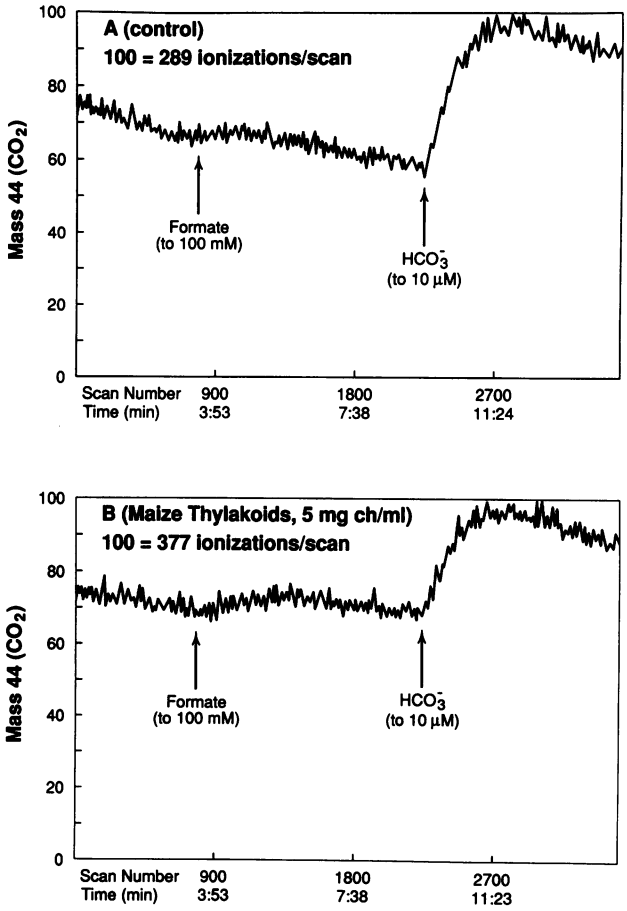

Figure 1. Absence of formate-induced release of bicarbonate from maize thylakoids. In A (control) 2 mL of the reaction mixture described in the Table I legend was placed in the reaction vessel attached to the mass spectrometer. Formate and bicarbonate were injected at the times indicated. In B, maize thylakoids (5 mg of Chl mL^{-1}) were also present. They were prepared as described in "Materials and Methods."

in solubility of the CO_2 already present in the reaction mixture. At 9.3 min, $NaHCO_3^-$ solution was injected to a final concentration of 10 μM. An obvious signal was recorded by the mass spectrometer. Clearly, the instrument had no difficulty measuring a concentration of 10 μM CO_2/HCO_3^- in the chamber.

The experiment was repeated with maize thylakoids (Fig. 1B). The concentration of Chl was high (5 mg mL^{-1}) to ensure that the HCO_3^- binding site concentration was enough for detection. In previous ^{14}C-HCO_3^- binding experiments (20), we determined that the number of HCO_3^- binding sites on maize thylakoids was at least one per 400 to 500 Chl molecules. Thus, the concentration of HCO_3^- binding sites in the reaction chamber was somewhat in excess of 10 μM.

After data acquisition was started, sodium formate (to 100 mM) was given to the thylakoids at 3.0 min. The total upward deflection of the baseline in the next several minutes was almost exactly the same as in the thylakoid-free controls. That is, formate failed to dislodge any appreciable HCO_3^- from the thylakoids. Again, at 9.3 min, $NaHCO_3^-$ solution was injected

to a concentration of 10 μM. As in the control, the mass spectrometer recorded a clear signal for the injected HCO_3^-. Similar results were obtained in 10 repetitions of this experiment. In several instances, it appeared that a trace more CO_2 signal was obtained from the thylakoids as compared to the controls. However, the added signal, when it appeared, was so small that it could not be accurately quantitated. Some experiments were done with spinach thylakoids and yielded similar results, except that, with spinach, at the high Chl concentrations used, a constant drift upward of the baseline CO_2 signal was observed. This was accompanied by a fairly rapid drift downward of the mass 32 (O_2) signal. Evidently, some respiratory processes were taking place. The experiments with maize, therefore, are more reliable.

The data displayed by the computer-controlled mass spectrometer is normalized to the maximum signal obtained during the acquisition period. The maximum signal varies somewhat because the baseline changes from sample to sample. The maximum signal is given at the upper left of each trace presented in Figure 1. Knowing this, one can easily calculate that the signal obtained by injection of 10 μM HCO_3^- into the thylakoid suspension was exactly the same as when injected into the control. This is important because it means that thylakoids under these conditions are not a sink for CO_2/HCO_3^- due, perhaps, to a very large number of low-affinity binding sites. In other words, if formate had dislodged bicarbonate from photosystem II, the anticipated CO_2 signal could not have been masked by the thylakoids themselves. Moreover, it was shown in previous experiments (14) that washing $H^{14}CO_3^-$-loaded thylakoids with formate at low pH does, in fact, remove virtually all $H^{14}CO_3^-$ from the membranes. The $H^{14}CO_3^-$ did not become "trapped" inside due, perhaps, to the influence of some local domains. Clearly, in the experiments described here, a CO_2 signal should have been observed upon formate treatment, but was not. The conclusion is that formate must have bound to empty sites on photosystem II.

DISCUSSION

There are now at least three lines of evidence that bound bicarbonate is not a requirement for photosystem II activity. (a) The binding constant of 80 μM CO_2/HCO_3^- is much too high (13, 19). At acid pH, in air-equilibrated solutions, most of the binding sites for HCO_3^- will be empty. Yet it is unnecessary to add supplementary HCO_3^- to obtain high rates of electron transport activity except in the presence of inhibitory anions. (b) Untreated chloroplasts, showing normal rates of electron flow, are inhibited by exogenous HCO_3^- when silicomolybdate is used as an electron acceptor (2, 8). If HCO_3^- was already present, to give normal rates of electron flow, exogenous HCO_3^- should have no effect. Clearly, in these experiments, added HCO_3^- became bound to *empty* sites. The sites involved showed a binding constant of about 80 μM CO_2/HCO_3^- in the absence of formate (8). Curiously, in this case, the binding of HCO_3^- led to an inhibition, not a stimulation, of electron flow rates. This is likewise difficult to explain if bound HCO_3^- is required for photosystem II activity. (c) In the present work, no CO_2/HCO_3^- was released when chloroplasts, showing normal rates of electron flow, were treated with formate. From this direct measurement, it is concluded that formate binds to empty sites, not ones that have bicarbonate already present. Thus, three very different approaches and experimental protocols all yield the same conclusion. Inhibitory anions do not normally replace endogenous bound HCO_3^- from photosystem II but bind to empty sites. Added HCO_3^- simply relieves the inhibition. The existence of empty anion binding sites does not normally impose a rate-limiting step in the Hill reaction under saturating continuous light.

In view of the present evidence, the term "HCO_3^--depleted chloroplasts" used to mean formate-pretreated or anion-inhibited chloroplasts, is incorrect. As suggested previously (8), less misleading terminology should be adopted.

For some time it has been suggested (4, 11, 15, 21, 22) that bicarbonate stimulates (*i.e.* fails to inhibit) photosystem II because it can act as a proton donor in the plastoquinone $(Q_B)^-$ reducing reactions. The data shown in this work do not argue against this mode of action for HCO_3^- when it is present. The results do suggest, however, that, if bicarbonate acts this way, another source of protons must be available when the anion binding sites are empty. Indeed, this must be the usual or normal source of protons.

Although it seems almost certain that HCO_3^- is not required for photochemical activity at the K_d 80 μM binding site, it is still possible that trace amounts of "free" CO_2/HCO_3^- could have some direct role in electron transport. This suggestion is consistent with the findings of Good (5) and Eaton-Rye *et al.* (3) that very small stimulation of Hill reaction rates can be observed with added CO_2/HCO_3^- in the absence of formate or large amounts of inhibitory anions. In neither of these studies were CO_2/HCO_3^- concentration curves generated, so the K_d 80 μM site may not be involved. This supposition is weak, however. In every case to date where a CO_2/HCO_3^- concentration study has been done (see, for example, ref. 8), a dissociation constant of about 80 μM has been found. The results do not, in any case, prove that a requirement for CO_2/HCO_3^- exists, since the effects were small and other interpretations of the data are possible. The pretreatments were long and, in the experiments of Eaton-Rye *et al.* (3), resulted in up to 80% irreversible damage. It is therefore possible that HCO_3^-, when present, can protect photosystem II activity (as can formate [3]), or that HCO_3^- can stimulate, slightly, some photosystem II reactions that are not normally rate-limiting. HCO_3^- could, therefore, have an effect without being a requirement for photosystem II activity, or even a major regulatory factor.

There are also experimental results from *in vivo* systems that are worth noting. Ireland *et al.* (7) concluded that varying the CO_2 levels over a physiological range around wheat and maize leaves could directly affect photosynthetic electron transport. With the green alga *Chlamydobotrys stellata*, Mende and Wiessner (10) showed that, within 15 min of removal of CO_2, photosystem II is inhibited on the electron donor side. A CO_2 deprivation time of 2 h resulted in an inhibition on the electron acceptor side as well. The results could not be repeated with *Chlorella*. One might conclude from the observations of these authors that CO_2/HCO_3^- could have some direct role in photosystem II electron transport. It

is possible, however, that the results reflect an indirect effect of CO_2/HCO_3^- on anion levels within the chloroplast. Under CO_2/HCO_3^- deprivation, small molecules, such as formate, acetate, glyoxylate, etc., might build up to inhibitory concentrations. Removal and readdition of CO_2, even at air levels, could also have marked effects on stromal pH, as shown by Oja *et al.* (12). Distinguishing between such indirect effects and those due to binding of CO_2/HCO_3^- to photosystem II in living systems presents a challenge. Still, the results are interesting and may reflect a true *in vivo* CO_2/HCO_3^- effect.

In my opinion, there is now clear evidence that HCO_3^- is not required for photosystem II activity at the K_d 80 μM binding site. There are other, perhaps more equivocal, data that CO_2/HCO_3^- may still play some direct role in electron transport. One can therefore pose the question, does CO_2/HCO_3^- have a second more fundamental role in photosystem II apart from its anion-induced action at the K_d 80 μM sites? For an affirmative response, CO_2/HCO_3^- must be shown to have large, unique, direct effects at concentrations significantly below 80 μM and in the absence of inhibitory anions. In effect, efforts to establish a CO_2/HCO_3^- requirement or regulatory role in photosystem II must start at the beginning.

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