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Forms of Dissolved Carbon Dioxide Required for Photosystem II Activity in Chloroplast Membranes[1]

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ABSTRACT

High concentrations of both bicarbonate and formate inhibit photosynthetic O_2 evolution at pH 8.0. At this pH, only 2.4% of the total dissolved carbon dioxide exists as CO_2. At pH 7.3, where 11% of the total dissolved carbon dioxide exists as CO_2, HCO_3^- no longer inhibits. While formate still inhibits O_2 evolution at pH 7.3, its effect can be partially overcome if CO_2 is also present. The rate of binding of added ^{14}C-labeled inorganic carbon is nearly 10-fold more rapid when the internal pH of thylakoid membranes is at 6.0 than when it is at 7.8. These observations suggest that CO_2, not HCO_3^-, is initially bound to the photosystem II reaction center and that the location of the binding site is on the inside thylakoid surface. However, additional data presented here suggest that, after binding, CO_2 is hydrated to $HCO_3^- + H^+$ in a pH-dependent reaction. Two possible explanations of the "bicarbonate effect" are presented.

It is established that PSII activity in chloroplast membranes requires catalytic amounts of dissolved CO_2 (11, 12, 15). This substance, denoted as C',[2] is bound to the reaction center complex (9). It has not been determined with certainty if CO_2 or HCO_3^- is the active form. The ligand interacts with the reaction center in a dynamic fashion (10). It is released very briefly in light, possibly when electrons are passed from the reaction center to PC, but is bound again in a subsequent "dark" reaction. To understand this cyclic binding and release of the ligand and to formulate an explanation of the "bicarbonate effect," it is necessary to know the form of inorganic carbon which is taken up, used and released by the reaction center. Early experiments indicated that HCO_3^- was, in fact, the "active form" (3, 6, 12). This conclusion, however, remained tentative.

A previous study (10) showed that high concentrations of formate can, in the light, progressively inhibit O_2 evolution. It does this by inhibiting the rebinding of C' released from the PSII reaction centers in the light. The present study was done to see if high concentrations of exogenous HCO_3^-, or CO_2, could nullify the effect of formate. Other studies were then done to see if the binding of radioactive $^{14}C'$ would be influenced by pH in the same manner as is the ratio of CO_2 to HCO_3^- in solution.

MATERIALS AND METHODS

Maize (*Zea mays* L.) plants were grown in soil under artificial light (40-w fluorescent lamps; 14/10 h light/dark photoperiod) at room temperature and harvested 8–14 days after planting. After harvesting, the leaves were ground for 30 s in a Waring Blendor set at maximum speed. This grinding regime will yield predominantly mesophyll grana but no attempt was made to completely exclude bundle sheath chloroplasts. The isolation medium contained 0.4 M sucrose, 0.01 M NaCl, and 0.05 M Na-phosphate (pH 6.8). All operations were done at near freezing temperatures. After grinding, the chloroplast slurry was filtered through four layers of cheesecloth and the filtrate was centrifuged for 1 min at 200*g*. The supernatant suspension was poured into clean tubes and centrifuged for 5 min at 1,500*g*. The resulting supernatant fluid was discarded. The chloroplasts comprising the pellet were then osmotically shocked by suspension in sucrose-free isolation medium. The broken chloroplasts were collected by centrifugation at 1,500*g* for 5 min. The resulting pellet was resuspended in a small amount of isolation medium, divided into portions, placed in a freezer and kept at −80 C. The chloroplasts were thawed just before use.

To deplete chloroplasts of endogenous C', thawed grana were suspended in depletion medium which contained 0.175 M NaCl, 0.1 M sodium formate, and 0.05 M Na-phosphate (pH 5.0). The Chl concentration was about 50 μg/ml. The room-temperature suspension was allowed to stand for 10 min, then it was centrifuged. The thylakoid membranes, now depleted of C', were resuspended in a small amount of depletion medium and kept on ice until used (see ref. 9).

Ferricyanide-supported O_2 evolution was monitored with a Rank Brothers, Clark-type electrode. The light source was a Sylvania 150-w projector lamp and the light was focused by a Prado slide projector. The incident light intensity of white light was 500 w/m^2.

The rate of binding of radioactive C' to PSII reaction centers was measured as follows. Broken chloroplasts were depleted of endogenous C' by the procedure given above. They were then collected by centrifugation and resuspended in a small amount of depletion medium. In some experiments the chloroplasts were suspended in other media as noted in the figure legends. Twenty μl of the concentrated chloroplast suspension were injected into 0.5 ml reaction medium which contained, unless otherwise stated in the figure legends, 0.1 M Na-phosphate (pH as specified), 0.01 M NaCl, 0.4 M sucrose, 0.33 mM $NaH^{14}CO_3$ (10 μCi), and 0.1 mM $K_3Fe(CN)_6$. Although experiments were done in dim room light, ferricyanide was routinely included in the reaction media to keep electron carriers, particularly B (2, 14) in the oxidized state. This appears to be an important factor in the binding of exogenous C' (10). The radioactive label was given to the chloroplasts in pure form, that is >97% $^{14}C'$. The concentration was kept low (0.33 mM) so that even after 2 min of incubation of $^{14}C'$ with the grana under optimum conditions, about 30% or less of the reaction centers were labeled. Higher concentrations of $^{14}C'$ would quickly saturate the reaction centers and the rate of binding would be difficult to measure.

The reaction media were all freed beforehand of dissolved CO_2

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[2] Abbreviations: C': CO_2 or HCO_3^-, when neither one can be specified; PQ: plastoquinone; PS_{II}: photosystem II reaction center complex; B: negative charge accumulator on the reducing side of photosystem II.

either by boiling them or by prolonged bubbling of pure N_2 through the solution. After 0.5 ml of the reaction medium was placed in each of a series of test tubes, 0.5 ml of mineral oil was placed in the tube to "cap" the solution. This was necessary to prevent loss of $^{14}CO_2$ when the pH of the reaction medium was low and to prevent the entry of atmospheric CO_2. Chloroplast suspensions and $NaH^{14}CO_3$ solutions were injected through the mineral oil.

After chloroplasts and radioactive label were mixed in the stirred reaction medium, the binding of $^{14}C'$ could be stopped at any time. This was done by injecting into the reaction medium 2.5 ml of ice-cold solution which contained 0.05 M Na-phosphate, 0.2 M NaCl and 0.02 M $NaHCO_3$ (unlabeled). The high concentration of unlabeled C′ diluted the $^{14}C'$ by a ratio of 303:1. Thus any continued binding of ligand would be reduced in $^{14}C'$ by this ratio. If, at the time the arresting solution was added, 10% of the reaction centers had bound $^{14}C'$, only an additional 0.3% could contain $^{14}C'$ when all the centers secured a ligand. This small amount of additional binding would also occur in the zero time control (see below) and could be subtracted from the total $^{14}C'$ bound. The pH of the arresting solution was such that the final pH was at least 9.0. It was kept basic because it was shown previously (Fig. 2 and Table II in ref. 9), that added C′ will not exchange with endogenous ligand at pH greater than about 7. To confirm that the unlabeled C′ in the arresting solution did not exchange with $^{14}C'$ bound during the incubation period, samples were allowed to incubate with pure $^{14}C'$ until all their binding sites were taken (one $^{14}C'$ bound per 300–400 Chl molecules, 9). Then arresting solution was given to some, while controls were given similarly buffered solution but without unlabeled $NaHCO_3$. Both treatments yielded samples bearing the same number of dpm/mg Chl. Thus the arresting solution used in these studies does not remove previously bound $^{14}C'$, but ensures that the $^{14}C'$ bound after addition will be a small per cent of the total.

For each experiment a zero time control was obtained. The binding of $^{14}C'$ was stopped immediately after mixing chloroplast and label. The activity measured from these zero time controls was subtracted from all other samples and thus eliminated such errors as $^{14}C'$ bound after addition of the arresting solution, background counts, "trapped" label and luminescence. The value subtracted was rarely more than 10% of the total observed after 2 min.

After the binding of $^{14}C'$ was stopped, the chloroplasts were collected by centrifugation. To eliminate unbound $^{14}C'$, the chloroplasts were then washed three times in a solution which contained 0.05 M Na-phosphate (pH 7.0), 0.01 M NaCl, and 0.3 M sucrose. The final pellet was resuspended in 0.05 M glycine solution at pH 9.5 which contained 4% (v/v) Triton X-100. The chloroplasts were allowed to dissolve in the dark for 30 min. Aliquots were placed in scintillation fluid made up of toluene, Triton X-100, and water in volume proportions of 1:0.5:0.16, respectively. The fluid also contained 4 g/l Omnifluor (New England Nuclear). Radioactivity was measured with a Beckman liquid scintillation counter model LS8000.

The method outlined above gave very reproducible results and, if the concentration of added $^{14}C'$ was kept low, binding was often almost directly proportional to time for at least 2 min (Fig. 2).

RESULTS

Reversal of Bicarbonate and Formate Inhibition of O_2 Evolution by CO_2. Chloroplasts not previously depleted of bound C′ were placed in the reaction vessel of a Clark-type O_2 electrode apparatus. It was shown previously (10) that PSII reaction centers will lose their bound C′ in the light if electron transport can take place and high (0.1 M) concentrations of formate are present. Apparently formate competitively inhibits the rebinding of the released ligand. In the light, I observed a progressive decrease in O_2 evolution as PSII reaction centers lose C′ and are turned off. In the experiment reported here it was supposed that a high concentration of exogenous inorganic carbon would negate the action of formate by replacing the endogenous ligand as it was lost. This supposition proved correct only when the pH of the reaction medium was low enough to ensure the presence of some CO_2.

When control chloroplasts were suspended in a reaction medium at pH 8.0 but which contained neither inorganic carbon nor formate, they gave off O_2 in the light as shown in Figure 1, curve A. When 0.05 M inorganic carbon (97.6% HCO_3^-, 2.4% CO_2) was also present, O_2 evolution was inhibited as shown by curve B. When 0.1 M sodium formate was present, even greater inhibition of oxygen evolution was observed (curve C). Maximum inhibition was observed when both inorganic carbon and formate were present together (curve D).

When this same experiment was repeated with a reaction medium at pH 7.3, quite different results were observed. Control chloroplasts given neither inorganic carbon nor formate evolved O_2 as shown in Figure 1, curve E. Added inorganic carbon (0.05 M, 89% HCO_3^-, 11% CO_2) had no effect on O_2 evolution (curve F) compared to the control. When 0.1 M sodium formate was present (curve H), O_2 evolution was inhibited just as it was at pH 8.0. At pH 7.3, however, the inhibition by formate was at least partially overcome by the simultaneous presence of inorganic carbon (curve G).

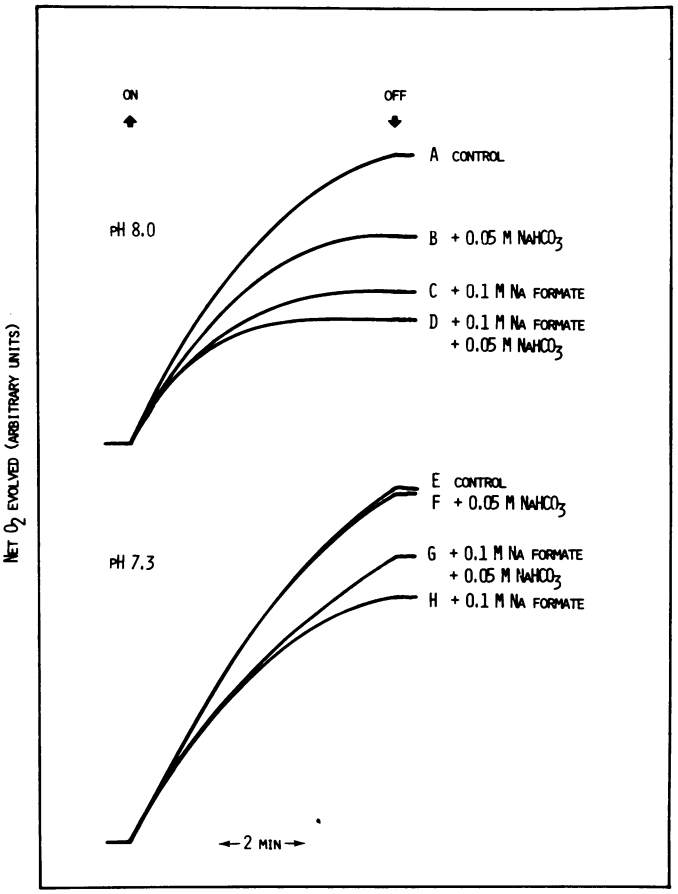

FIG. 1. The ability of CO_2 to overcome formate and bicarbonate inhibition of O_2 evolution. Chloroplasts, undepleted of C′ were suspended in a reaction mixture which contained 0.1 M Na-phosphate, 0.2 M NaCl, 0.5 mM $K_3Fe(CN)_6$, 40 μg Chl/ml, and the additions indicated in the figure. The final pH was either 8.0 or 7.3 as indicated. The light intensity was 500 w/m^2 and the temperature was 20 C.

These results can best be understood by considering the effects of pH on the relative amounts of CO_2 and HCO_3^- present in solution. In going from pH 8.0 to 7.3, the amount of HCO_3^- changes very little, a decrease from 97.6 to 89%. The concentration of CO_2, on the other hand, increases more than 4-fold, from 2.4 to 11%. Evidently it is this higher concentration of CO_2 at pH 7.3 compared to that at 8.0, which prevented the inhibition of O_2 evolution by HCO_3^- and partially reversed the inhibition by formate.

It is suggested, therefore, that CO_2, not HCO_3^- is the form of C' taken up initially by the reaction center. It also seems that HCO_3^-, like formate, will inhibit the binding of CO_2, perhaps competitively.

BINDING OF $^{14}CO_2$ *versus* $H^{14}CO_3^-$

Effect of External pH. If CO_2 is bound initially to PSII reaction centers, it might be expected that the rate of binding of $^{14}C'$ to C'-depleted thylakoids would be greater at lower pH where CO_2 predominates, than at higher pH where HCO_3^- is the predominant form. Chloroplasts were depleted of endogenous C', collected by centrifugation and then resuspended in a small amount of depletion medium. The pH of the depletion medium was 5.0 and it contained 0.1 M sodium formate. When this chloroplast suspension was injected into reaction mixtures at either pH 7.8, 6.8, or 6.0 (which, therefore, contained, 4, 27, or 70% CO_2, respectively), the rate of binding of $^{14}C'$ for the first 2 min was pH-independent (Fig. 2). Clearly the pH of the external medium could not control the rate of binding of $^{14}C'$ despite large variation in the CO_2/HCO_3^- ratio. This unexpected result suggested that the conditions inside the thylakoid vesicles might be critical in controlling the rate of binding, since the internal conditions were initially the same regardless of external pH.

Effect of Internal pH. The $^{14}C'$ binding experiment discussed above was repeated with the following variation. Chloroplasts depleted of C' were injected into reaction mixtures at either pH 7.8, 6.8, or 6.0 but which did not contain radioactive inorganic carbon. After a 5-min equilibration period, $NaH^{14}CO_3$ was injected into the samples and the rate of binding was thereafter measured (Fig. 3). Compared to nonequilibrated chloroplasts (Fig. 2) the rate of binding to equilibrated chloroplasts increased at pH 6.0, remained about the same at pH 6.8 and decreased dramatically at pH 7.8. It appears that after the chloroplasts equilibrate and their internal pH equaled the external pH, a 10-fold more rapid binding is observed at pH 6.0 than at 7.8. This is consistent with the notion that CO_2, not HCO_3^-, is initially bound to PSII reaction centers. It is also apparent that CO_2 must be binding on the inside surface of the thylakoid membrane. As a result of equilibration, however, a second internal factor, the formate concentration, also changes.

Effect of Removing Internal Formate. Chloroplasts were depleted of endogenous C', collected by centrifugation, and resuspended for 10 min in depletion medium from which sodium formate was omitted. After a second centrifugation the pellet was resuspended in formate-free depletion medium. The chloroplast suspension was then injected into reaction medium at pH 7.8, 6.8, or 6.0 and the rate of $^{14}C'$ binding was measured. The chloroplasts were not equilibrated beforehand so the internal pH of the thylakoids was initially at 5.0 but in this case no formate was present (Fig. 4). With internal formate absent, the rate of binding of label is about twice as fast with external pH at 6.0 or 6.8 compared to when formate is present (see Fig. 2). This is consistent with the hypothesis that formate inhibits the binding of CO_2. However, the absence of formate does not, as one would expect, also stimulate $^{14}C'$ binding when the external pH is 7.8 (compare Figs. 2 and 4). The results shown in Figure 4 indicate that the chloroplasts must

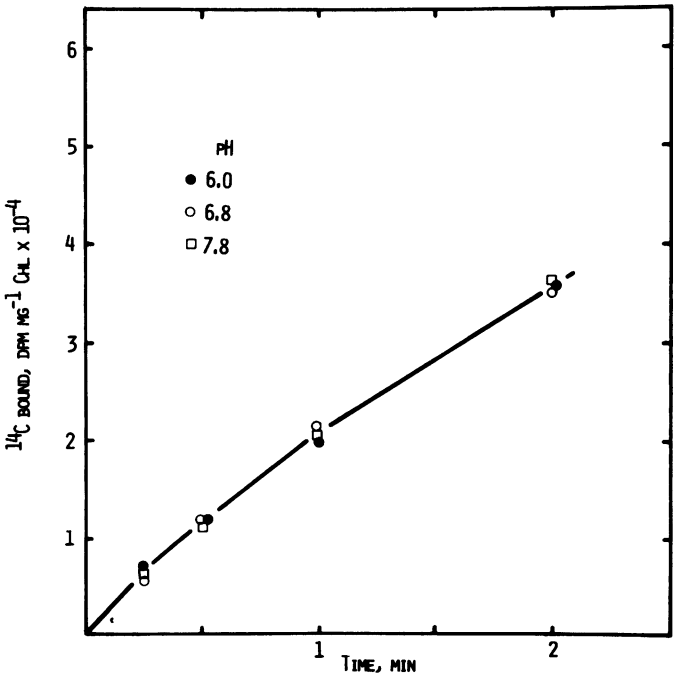

FIG. 2. The initial rate of binding of $^{14}C'$ to chloroplast grana at various pH of the external reaction medium. Chloroplasts were depleted of C', collected by centrifugation and resuspended in a small amount of depletion medium. Twenty μl of chloroplast suspension (50 μg Chl) were injected into 0.5 ml of reaction medium which contained 0.1 M Na-phosphate, 10 mM NaCl, 0.4 M sucrose, 0.1 mM $K_3Fe(CN)_6$, and 0.33 mM $NaH^{14}CO_3$ (10 μCi). After incubation at 27 C for the times indicated, binding was stopped and the amount of bound $^{14}C'$ was determined.

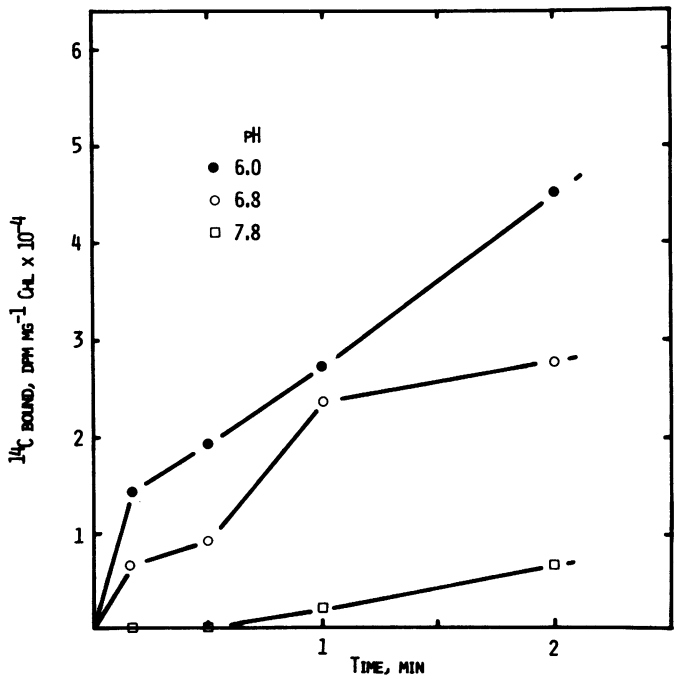

FIG. 3. The initial rate of binding of $^{14}C'$ to chloroplast after 5 min equilibration time in reaction mixtures of various pH. Chloroplasts depleted of C' were collected by centrifugation and resuspended in a small amount of depletion medium. Twenty μl of chloroplast suspension were injected into the reaction mixture (see legend Fig. 2) in which $NaH^{14}CO_3$ was omitted. After 5 min equilibration at 27 C, $NaH^{14}CO_3$ (10 μCi) was injected to a final concentration of 0.33 mM. Binding was then stopped after the indicated times and the amount of bound $^{14}C'$ was measured.

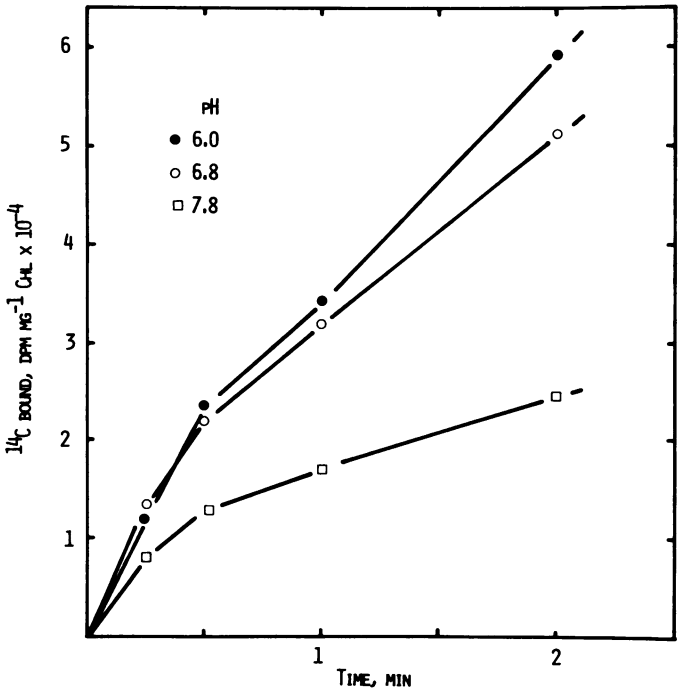

FIG. 4. The initial rate of binding of $^{14}C'$ to chloroplast grana at various external pH when formate is absent from the interior of the thylakoids. Chloroplasts were depleted of bound C', collected by centrifugation and then resuspended in 5 ml formate-free depletion medium which then contained only 0.1 M Na-phosphate (pH 5.0) and 0.175 M NaCl. After 10 min at room temperature the chloroplasts were collected again by centrifugation and resuspended in a small amount of formate-free depletion medium. Twenty μl of suspension was then injected into reaction medium as the experiment described for Figure 2 was repeated.

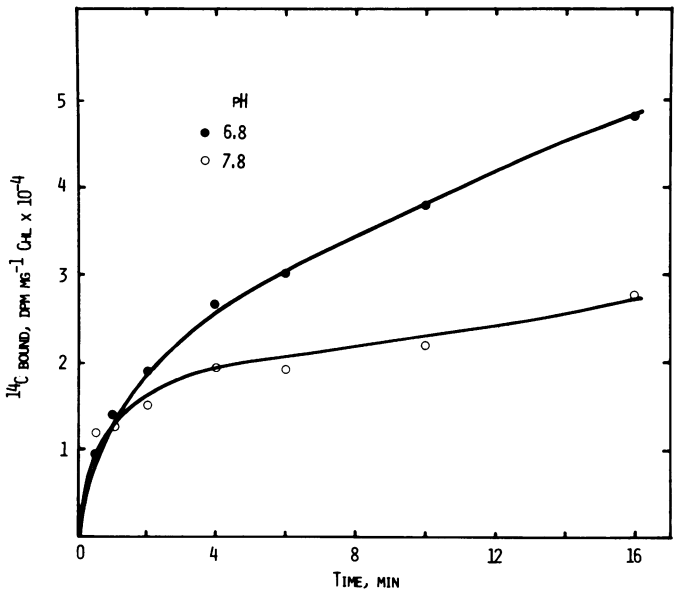

FIG. 5. The binding of $^{14}C'$ to chloroplast grana as a function of time. Conditions were identical to those described in the legend of Figure 2 except that, here, longer incubation times were allowed and the temperature was 20 C.

equilibrate with respect to transmembrane pH, to some degree at least, during the first 2 min of binding. Otherwise the rate of binding would not be lower at external pH 7.8 than at 6.0 or 6.8. Why does this pH equilibration have no effect when formate is present as shown by the results in Figure 2? I propose that formate has a greater ability to inhibit C' binding when the pH is low. This increased inhibitory ability must exactly compensate for the greater CO_2 present at low internal pH. The observed rate of $^{14}C'$ binding in the presence of formate is the same regardless of the change in internal pH. In other words, the absence of internal formate allows the pH effect to be observed.

Long-term Binding of $^{14}C'$. The effect of pH on the rate of binding of $^{14}C'$ also becomes evident when experiments are done over longer time periods. The same experiment done to obtain the results shown in Figure 2 was extended from 2 to 16 min (Fig. 5). Here, only the effects of external pH 7.8 *versus* 6.8 were observed. At both external pH 7.8 and 6.8 the rate of binding is clearly biphasic. For about 2 min the chloroplasts show a high rate of $^{14}C'$ binding and, like the results shown in Figure 2, the rate is nearly the same at pH 7.8 as at 6.8. After 2 min, as pH and formate concentration equilibrate across the thylakoid membranes, the rate of $^{14}C'$ binding declines. At pH 7.8, where only 5% of the total inorganic carbon is in the form of CO_2, the rate of binding between 2 and 16 min is hardly measurable. In contrast, at pH 6.8, where CO_2 is 27% of the inorganic carbon, the rate of binding between 2 and 16 min, although slower than the initial rate, proceeds steadily for the entire time. Again these data are consistent with the proposal that CO_2 is bound and that the internal thylakoid conditions control the rate of binding.

Long-term Binding of $^{14}C'$ as a Function of pH. Not all observations fit the simplified scheme of CO_2 binding which has been presented thus far. A more complete study of long-term binding of $^{14}C'$ as a function of pH revealed complications.

Chloroplasts depleted of endogenous C' were injected into reaction mixtures ranging from pH 5.0 to 8.0. The mixtures either contained high concentrations of sodium chloride plus sodium formate, or sucrose (see legend, Fig. 6). The suspensions were incubated for 12 min, then binding was stopped and the amount of bound $^{14}C'$ was assayed. In the absence of high NaCl and sodium formate concentrations (Fig. 6, top curve), the maximum amount of $^{14}C'$ bound after 12 min is at about pH 6.4 to 6.8, slightly above the pK_a of carbonic acid (approximately 6.4). The amount of $^{14}C'$ bound declines more sharply below than above the maximum. The decline in the amount of $^{14}C'$ bound above pH 7 can be explained on the basis of a reduced CO_2 concentration such that even after 12 min, not all the reaction centers are filled (see Fig. 5). The decline in the amount of $^{14}C'$ bound below pH 6.4 cannot be explained on the basis of reduced CO_2 concentration since it increases as the pH falls below the pK_a of carbonic acid. It is proposed therefore that the binding of CO_2 is reversible below, but not above, the pK_a of carbonic acid. Above pH 6.4 the CO_2 which binds to the reaction center is increasingly stabilized by hydration to $HCO_3^- + H^+$. In this ionic form, the bound ligand appears stable. It can not be lost, nor will it exchange with free HCO_3^-. The hydration and stabilization of CO_2 is especially important when binding occurs in the presence of high chloride and formate concentrations (Fig. 6, lower curve). Here one sees that the pH optimum for binding is shifted to 8.0 or above. Again, as in Figures 2 and 4, formate has less apparent ability to inhibit CO_2 binding at pH above the pK_a of carbonic acid. Clearly the situation must be viewed in dynamic terms. Formate inhibits the binding of CO_2. If the binding is reversible below the pK_a of carbonic acid, formate can inhibit the binding each time CO_2 attempts to bind back to the reaction center. Above the pK_a of carbonic acid, another situation exists. Formate still inhibits the rate of binding of CO_2 but if the ligand can bind to reaction center once, the CO_2 will be permanently "captured" by hydration to $HCO_3^- + H^+$. This could account for the shift in the pH optimum for binding of $^{14}C'$ from 6.6 to 8.0 when formate is present.

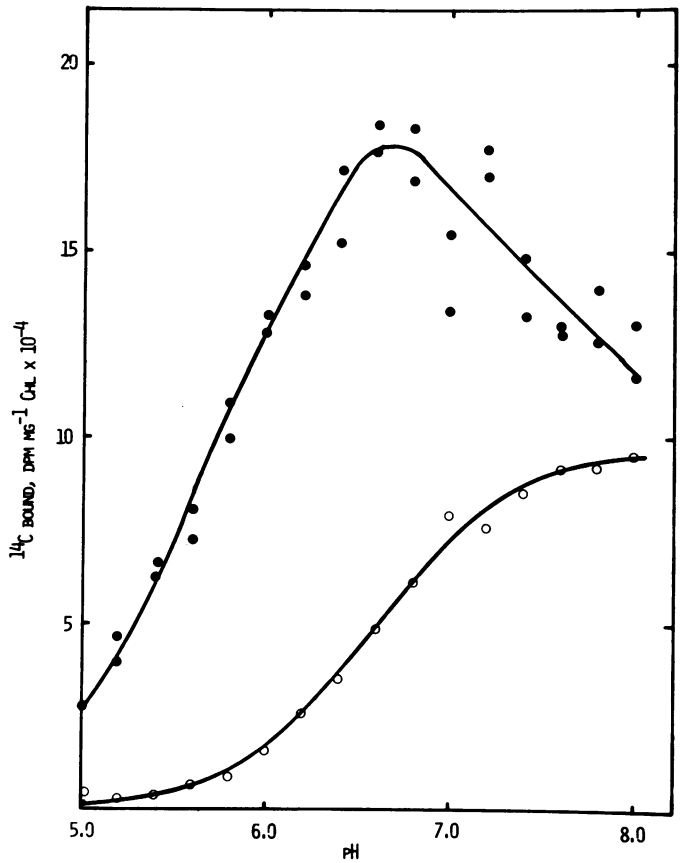

FIG. 6. The amount of $^{14}C'$ bound to chloroplasts after 12 min incubation time as a function of pH. Chloroplasts were depleted of C', collected by centrifugation and resuspended in a small amount of depletion medium. Twenty μl of the chloroplast suspension was injected into reaction mixture at 28 C. The binding of $^{14}C'$ was stopped after 12 min. The reaction mixture contained, in addition to 0.1 M Na-phosphate, 0.33 mM $NaH^{14}CO_3$ (10 μCi), 0.1 mM $K_3Fe(CN)_6$, either (top curve) 0.01 M NaCl plus 0.4 M sucrose or (bottom curve) 0.175 M NaCl plus 0.1 M sodium formate.

DISCUSSION

In a previous publication (10) a set of reactions was proposed to describe the dynamic nature of the interaction between PSII and C' in the light. The information presented here can provide some detail to this working hypothesis. The following modifications are proposed:

$$PS_{II}B + CO_2 \underset{\text{formate or } HCO_3^-}{\rightleftharpoons} [PS_{II}B - CO_2] \quad (1)$$

$$[PS_{II}B - CO_2] + H_2O \underset{H^+}{\overset{OH}{\rightleftharpoons}} [PS_{II}B - HCO_3^-] + H^+ \quad (2)$$

$$[PS_{II}B - HCO_3^-] \xrightarrow{2\,h\nu} [PS_{II}B^{2-} - HCO_3^-] \quad (3)$$

$$[PS_{II}B^{2-} - HCO_3^-] \xrightarrow{PQ \rightarrow PQ^{2-}} PS_{II}B + CO_2 + OH^- \quad (4)$$

Here $PS_{II}B$ represents the PSII reaction center complex to which is attached B, the charge accumulator on the reducing side (2, 14).

In the first equation the reaction center, or probably a specific protein component of the reaction center, forms a complex with CO_2 when B is in the fully oxidized state (see ref. 10 for the rationale and supporting evidence). The complex formation is a "dark" reaction (9, 10) and is reversible. The forward reaction, toward the right, is favored by high CO_2 concentration and is inhibited by formate and bicarbonate which compete with CO_2 for the binding site. The reverse reaction, toward the left, is not inhibited by formate so the net effect of high concentrations of this anion will be to shift the equilibrium to the left, thus freeing CO_2. This is why formate must be added to the "HCO_3^--depletion medium" (9).

Once the reaction center-CO_2 complex is formed, CO_2 is hydrated to form HCO_3^- and H^+ as shown by equation 2. It is proposed that the reaction center itself acts as carbonic anhydrase in catalyzing this reaction. An important aspect of the reaction will be its reversibility and pH dependence. A forward direction, toward the right, will be favored at high pH (above 6.4 the pK_a of carbonic acid) while dehydration and then dissociation of the complex will be favored at low pH. I propose that the formation of HCO_3^- stabilizes the complex and that only as CO_2 can the ligand be lost or exchanged. This proposal is consistent with observations that labeled $^{14}C'$ will exchange with endogenous ligand only at pH below about 7 and explains why low pH is required in the depletion procedure (9). Formate will have no effect on reaction 2 in either direction. Considering reactions 1 and 2 together, it will appear that formate has less inhibitory effect on the rate of binding of CO_2 at high pH than at low (compare Figs. 2 and 4) while, in reality, it has less effect on the final or steady-state extent of binding at high pH (Fig. 6).

In the light, after formation of the HCO_3^- complex, the reaction center undergoes reaction(s) 3 where the charge accumulator B is first singly, then doubly reduced. After complete reduction of B, electrons are transferred to PQ as in reaction 4. This results in a splitting of the complex such that the ligand becomes momentarily free. Two of the products of reaction 4, the reaction center complex and CO_2, recombine immediately as in reaction 1. It is necessary to argue, here, why reaction 4 releases CO_2 plus OH^-, and not HCO_3^-. If HCO_3^- were released it must, in solution, take up a proton and dissociate into CO_2 and H_2O. Otherwise the reaction center, binding only CO_2, will not "see" the ligand. Reaction 1 could not take place and the cycle would be broken. The rate at which free HCO_3^- could react with a proton and dissociate spontaneously is slow, especially at high pH, occurring in 10–100 ms or longer (8). The broken and washed chloroplasts used in these studies do not appear to have the ability to speed this reaction (13). Since reaction centers can recover (and presumably rebind CO_2) in less than a millisecond following any photoact (1), the slow conversion of free HCO_3^- to CO_2 cannot be taking place. Thus CO_2, not HCO_3^-, must be released in reaction 4.

To summarize the scheme, the data presented here and in previous publications suggest a dark binding of CO_2 to PSII reaction centers followed by hydration and dissociation of the ligand to HCO_3^- and H^+. In the light, HCO_3^- is dehydroxylated to CO_2 and OH^-. The cycle repeats. Thus both forms of dissolved CO_2 play a role in the "bicarbonate effect" in PSII.

The scheme presented here is tentative. There is no direct evidence, for example, that CO_2 is released when electrons are transferred from B to PQ. Some recent work done in the author's laboratory seems to indicate that the release of CO_2 can be explained better with reference to reactions occurring on the O_2 evolving side of PSII. As more evidence becomes available, therefore, the scheme will probably require revision.

TWO HYPOTHESES

If the scheme presented above is correct, at least in a general sense, two hypotheses can be made to explain the C' requirement. Reaction 2 of the scheme results in the liberation of a proton whereas reaction 4 results in the liberation of an hydroxyl ion. If, by some yet unknown mechanism, these two events occur on opposite sides of the thylakoid membrane, then a pH gradient will be established. Here, C' is proposed to play a central role in the energy-conserving mechanism associated with PSII.

A second hypothesis, requiring a modified scheme has, in fact, already been offered. It has been proposed by Metzner (7) that HCO_3^-, rather than H_2O itself, is the immediate source of photo-

synthetically evolved O_2. This hypothesis would suggest that the balance of reactions 1 through 4 above should be written:

$$[PS_{II}B - HCO_3^-] + H^+ \xrightarrow{2\ h\nu} PS_{II}B^{2-} + CO_2 + 0.5\ O_2 + 2H^+$$

In this abridged scheme bicarbonate is split and electrons are passed to the charge accumulator B and on to PSI. O_2 and CO_2 are liberated. Water remains the ultimate source of both electrons and oxygen, but CO_2 is an essential catalyst. The same cycle of dark hydration and light dehydration of CO_2 would be required in this hypothesis as in the first.

The evidence is still insufficient to decide between these two hypotheses, or to reject both. It has been argued by Govindjee and Van Rensen (4) that HCO_3^- operates strictly on the reducing side of PSII. If this is true, then the first hypothesis offered here is favored. On the other hand the removal of bound C′ from grana membranes completely inactivates a large fraction of the PSII reaction centers (5, 10) and the reason for this inactivation has not yet been explained by reference to the reducing side of photosystem II alone. Thus the second hypothesis remains viable.

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