Biochimica et Biophysica Acta, 460 (1977) 511–522

BBA 47301

THE BINDING OF BICARBONATE IONS TO WASHED CHLOROPLAST GRANA*

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(Received November 26th, 1976)

SUMMARY

Radioactive labelling techniques show that isolated broken chloroplasts can take up HCO_3^- in the dark. There are two pools of binding sites for this ion on, or within, the thylakoid membranes. A smaller, high affinity pool exists at a concentration of one HCO_3^- bound per 380–400 chlorophyll molecules. Removal of HCO_3^- bound in this pool requires special conditions and results in greater than 90 % inhibition of oxygen evolution. The inhibition is fully reversed when HCO_3^- is added back. HCO_3^- bound in the small pool does not necessarily exchange with free HCO_3^- in the dark or in light. Evidence presented suggests that this site is very near the site of action of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea. A second, much larger, pool of HCO_3^- binding sites also exists in a concentration approaching that of the bulk chlorophyll. These sites have a much lower affinity for HCO_3^-, and their function has not yet been determined.

INTRODUCTION

HCO_3^- is now known to play a direct role in early photochemical reactions in photosynthesis [1–4]. HCO_3^- is necessary for maximal Photosystem II activity. Evidence suggests [2] that under certain conditions HCO_3^- is taken up by broken chloroplasts in the dark as a prior condition to activation of oxygen evolution during a ferricyanide-dependent Hill reaction. A quantitative estimate of HCO_3^- uptake by broken chloroplast fragments is therefore desirable to allow insight into possible modes of action of this ion. To obtain such an estimate, competitive binding and other studies were done with $H^{14}CO_3^-$ and maize chloroplast fragments.

METHODS

Maize (*Zea mays* L.) plants were grown in a greenhouse and harvested when

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; SiMo, sodium silico-12-molybdate.

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2–4 weeks old. The procedure used to isolate the chloroplasts has been described elsewhere [2]. During isolation, the chloroplasts were subjected to osmotic shock after which they were washed, frozen, and then thawed before use. The term "chloroplasts" hereafter refers to such broken chloroplast fragments.

The binding of HCO_3^- to chloroplast fragments was measured by radioactive labelling $H^{14}CO_3^-$ and liquid scintillation counting. The scintillation fluid contained toluene, Triton X-100 and water in volume proportions of 1 : 0.5 : 0.16, respectively. To this solution was added 4 g/l Omnifluor (New England Nuclear). The counting instrument was an LKB-Wallac 81000 liquid scintillation counter. The instrument was programmed to calculate disintegrations per min after external standardization thus correcting for color and chemical quenching.

The experimental protocol for the competitive binding studies was as follows: chloroplast fragments were suspended in reaction mixture (described in the legends) and provided with $H^{14}CO_3^-$, then incubated in the dark at 30 °C for 5 min. After incubation, the suspension was centrifuged at top speed in a clinical centrifuge for 15 min at 2 °C. HCO_3^- content of the supernatant was then determined. After drawing off the remaining supernatant completely, the grana-containing pellet was resuspended in 0.05 M glycine buffer at pH 9.5, containing also 4 % (v/v) Triton X-100. The chloroplasts were allowed to dissolve in the dark for 1 h. Aliquots were then measured for radioactivity.

Since chloroplast fragments have a finite volume, they unavoidably contained a large amount of unbound or "trapped" $H^{14}CO_3^-$ along with the bound $H^{14}CO_3^-$. Estimation of this trapped $H^{14}CO_3^-$ was carried out with controls which were provided an excess (200 mM) of unlabelled HCO_3^- prior to 5 min incubation to compete for binding sites along with the $H^{14}CO_3^-$. In these same controls, the pH was allowed to rise to 7.4 and they were also kept in ice. Both treatments reduce binding of HCO_3^- (see text) but have no effect on the amount of trapped $H^{14}CO_3^-$. The $H^{14}CO_3^-$ remaining in controls so treated was considered "trapped" and this amount could then be subtracted from the total $H^{14}CO_3^-$ found in the experimental chloroplasts to determine the amount of $H^{14}CO_3^-$ actually bound. The trapped $H^{14}CO_3^-$ varied between 60 and 100 % of the total, depending on treatment.

Some competitive binding experiments were attempted with spinach grana rather than maize. Unfortunately, the total volume of spinach chloroplasts was found to be about three times that of maize on a per unit chlorophyll basis. The amount of trapped $H^{14}CO_3^-$ in spinach grana was, therefore, much greater relative to the bound $H^{14}CO_3^-$ to the extent that consistent results for most experiments were difficult to obtain.

For reproducible and valid results in doing the competitive binding experiments it was necessary to maintain a constant pH regardless of the concentration of added $NaHCO_3$. To do so a series of buffered solutions were made such that addition of a given amount of $NaHCO_3$ raised the reaction mixture pH to between 6.5 and 6.6.

Ferricyanide-supported oxygen evolution was monitored with a Rank Brothers, Clark-type electrode. The light source was a Sylvania 500 W projector lamp focused by a Crestline slide projector. The beam passed through a Corning 3-66 orange cut-off filter and the intensity incident on the sample was 0.24 μeinsteins $\cdot$ $cm^{-2} \cdot s^{-1}$.

RESULTS

Competitive binding of $H^{14}CO_3^-$, a Scatchard plot

Chloroplast grana were provided with $H^{14}CO_3^-$ along with different concentrations of unlabelled HCO_3^- increasing from 0 to 50 mM. Competition therefore developed for a limited number of binding sites on or within the thylakoid membranes. After correction for trapped $H^{14}CO_3^-$ (see Methods), results are expressed in a Scatchard plot [5] shown in Fig. 1. Where a single binding site is involved (as with a purified enzyme binding one ligand per molecule) a Scatchard plot will yield a straight line with the intercept of the abscissa indicating the concentration of binding site. With chloroplasts, however, a curved line is seen indicating the presence in grana of more than one kind of binding site for HCO_3^- in terms of affinity. The sharp drop in the left portion of the curve shows a small pool of binding sites having a relatively high affinity for HCO_3^-. A rough estimate of the maximum size of this pool can be obtained by a line tangent to the upper section of the curve and intersecting the abscissa [6]. The dashed line so drawn in Fig. 1 intersects the abscissa at 3.5 μM and this represents the upper limit of the concentration of this binding site when the chlorophyll concentration is 1.1 mM.

The estimation of this pool size is here only approximate as a curved Scatchard plot is difficult to analyze precisely [6, 7]. Besides, the conditions of the experiment appeared later not to have been such as to produce optimal HCO_3^- binding. Nevertheless it appears from these preliminary data that the concentration of the high affinity binding site is the same as the concentration of Photosystem II reaction centers, assuming one such center per 300 chlorophyll molecules. A more precise determination of the size of the small pool will be presented later.

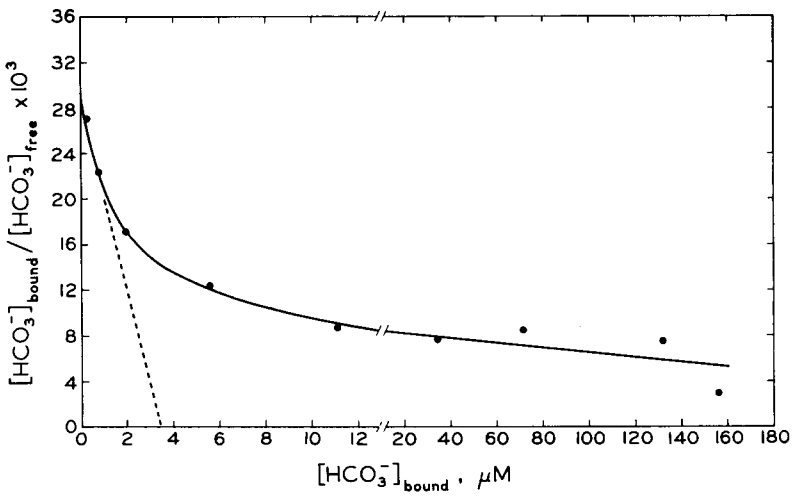

Fig. 1. Scatchard plot for the binding of HCO_3^- to chloroplast grana. The suspension contained 1.0 mg chlorophyll/ml (1.1 mM), 0.1 M sodium phosphate, pH 6.5, 0.2 M NaCl, 1.2 μCi $NaH^{14}CO_3$/ml. The concentration of unlabelled $NaHCO_3$ was varied from 0.0 to 50 mM. The grana were incubated for 5 min at 30 °C after addition of $H^{14}CO_3^-$. Each of the last four points is an average of eight separate sample measurements at different HCO_3^- concentrations.

The long tail of the Scatchard plot (Fig. 1) which approaches the abscissa very obliquely to the right indicates that there also exists in grana another quite large pool of relatively low affinity HCO_3^- binding sites. The size of this pool is difficult to determine since it cannot be saturated because of the limited solubility of HCO_3^-/CO_2 at the pH at which the experiment was done (6.5). However, the size must be greater than 160 μM when the chlorophyll concentration is 1.1 mM since this amount was actually observed bound under a non-saturating concentration of HCO_3^-. In other experiments (data not shown) more than twice this amount was observed bound. It seems reasonable therefore, that the concentration of this pool of binding sites may be near the concentration of bulk chlorophyll and could conceivably be higher. The relatively low affinity of these binding sites for HCO_3^- would mean that under normal physiological HCO_3^- concentrations, only a very small fraction of the sites would actually be occupied.

Further evidence that chloroplasts bind HCO_3^- was obtained from experiments in which an attempt was made to wash out the $H^{14}CO_3^-$ from previously loaded chloroplasts. Chloroplasts were provided $H^{14}CO_3^-$ and, after incubation, centrifuged. The ratio of dpm in the pellet (dpm_p) to the dpm in the supernatant (dpm_s) was determined. The grana were resuspended in buffered solution and again precipitated by centrifugation. Again the ratio dpm_p/dpm_s was determined. If the chloroplasts bind HCO_3^- strongly, the ratio dpm_p/dpm_s should increase after such washing.

Three concentrations of HCO_3^- were allowed in the original suspensions, 16 μM, 20 mM and 200 mM each containing the same amount of label (0.5 μCi). When 16 μM HCO_3^- is provided, most of the label will enter the small, high affinity binding pool (see left portion of the Scatchard plot, Fig. 1). When 20 mM HCO_3^- is provided, on the other hand, most of the label will be in the large, low affinity binding pool (right portion of the Scatchard plot). Thus by manipulating the concentration of unlabelled HCO_3^- we can divert the accompanying $H^{14}CO_3^-$ into either the large or small pool, though only predominantly, never completely. A final concentration, 200 mM, was chosen as the control. At this concentration, no binding of $H^{14}CO_3^-$ is expected, provided the pH is allowed to rise and the chloroplasts are kept cold, factors which will be discussed later.

The results of these experiments are shown in Table I. When chloroplasts are given 16 μM HCO_3^-, then precipitated, the ratio dpm_p/dpm_s averaged 0.015 (upper box). Resuspending the pellet in buffered wash solution and reprecipitating, the ratio dpm_p/dpm_s increased 16-fold to average 0.240. Clearly the HCO_3^-, once bound in the small, high affinity pool, tends to remain with the grana and cannot be washed out. The HCO_3^- that does appear in the wash supernatant is predominantly that which was merely "trapped" by the original pellet and would be expected to wash out.

When chloroplasts are given 20 mM HCO_3^- and similarly treated, the ratio dpm_p/dpm_s after the first centrifugation averages 0.013 (Table I, middle box). After washing, the ratio increases only slightly to 0.020. This indicates that HCO_3^- bound by the large, low affinity pool can be removed to some extent by a single washing.

In control chloroplasts (Table I, lower box) given 200 mM HCO_3^- and treated so as to minimize binding of HCO_3^-, the ratio dpm_p/dpm_s after the first centrifugation averages about 0.0095. After a single washing, the chloroplast pellet contains so few dpm over background that accurate determination was difficult. In no

TABLE I

DISTRIBUTION OF $H^{14}CO_3^-$ BETWEEN CHLOROPLAST PELLET AND SUPERNATANT BEFORE AND AFTER ONE WASHING

Chloroplasts (0.19 mg chlorophyll) were suspended in 1 ml of the solution indicated containing also 0.5 μCi $NaH^{14}CO_3$, incubated for 5 min at 30 °C and centrifuged. After determining dpm in the pellet (dpm_p) and supernatant (dpm_s) the pellet was resuspended in 1 ml of wash buffer at room temperature containing 0.1 M sodium phosphate, pH 7.0, 0.01 M NaCl, 0.3 M sucrose. The chloroplasts were recentrifuged and dpm again determined in the supernatant and pellet. Duplicate experiments are shown. The results are normalized to the same dpm in the first supernatants.

Chloroplast suspension medium		dpm_s	dpm_p	dpm_p/dpm_s
16 μM $NaHCO_3$	(1)	50 000	797	0.016
0.1 M sodium phosphate, pH 6.0	(2)	50 000	711	0.014
0.2 M NaCl				
Wash buffer	(1)	685	176	0.257
	(2)	622	139	0.224
20 mM $NaHCO_3$	(1)	50 000	668	0.012
0.1 M sodium phosphate, pH 6.2	(2)	50 000	656	0.013
0.2 M NaCl				
Wash buffer	(1)	704	14	0.020
	(2)	656	13	0.020
200 mM $NaHCO_3$	(1)	50 000	555	0.010
0.1 M sodium phosphate, pH 7.4	(2)	50 000	467	0.009
0.01 M NaCl				
Wash buffer	(1)	578	< 4	< 0.007
	(2)	446	< 4	< 0.009

case, however, was the ratio dpm_p/dpm_s ever observed to exceed that seen after the first centrifugation. Thus, in the controls, all the label accompanying the original pellet was merely trapped and therefore removed completely into the wash supernatant. Such controls provide us with the means of determining the ratio of bound vs. trapped $H^{14}CO_3^-$ as discussed in Methods.

After a second washing (data not shown) no label at all remains in control chloroplasts given 200 mM HCO_3^- initially, and very little in those given 20 mM HCO_3^-. Those chloroplasts given only 16 μM HCO_3^-, however, retain the $H^{14}CO_3^-$ without diminution through a second, third and even a fourth washing. This is further indication that once HCO_3^- is bound in the small pool, it cannot be dislodged again without special treatment. Such treatment will be discussed in a following section.

Effect of pH on high affinity binding of $H^{14}CO_3^-$

The pH of the chloroplast suspension medium is very important in allowing exchange in the dark between endogenous HCO_3^- and added $H^{14}CO_3^-$. Chloroplasts were suspended in incubation medium containing 33 μM $NaH^{14}CO_3$, a concentration at which most of the label will enter the small, high affinity binding pool. After incubation, the suspensions were centrifuged. At a pH below 7.0, the incubation medium lost some HCO_3^- as CO_2 (as much as half at very low pH), since the experiment could not be done under sealed conditions. To overcome this problem, immediately after centrifugation, the dpm in the supernatant, here designated dpm_{free} was

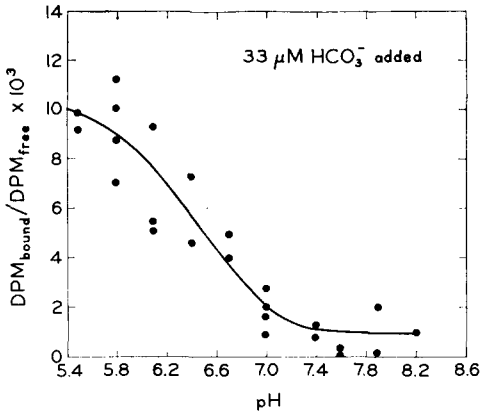

Fig. 2. Binding of $H^{14}CO_3^-$ to the high affinity site in chloroplast grana as a function of pH. The suspension contained 384 μg chlorophyll/ml, 0.1 M sodium phosphate, 0.2 M NaCl, 33 μM $NaH^{14}CO_3$ (1.2 μCi/ml). The pH was varied by addition of appropriate amounts of NaOH.

measured as well as the $H^{14}CO_3$ bound in the pellet, designated dpm_{bound}. Plotting the ratio dpm_{bound}/dpm_{free} as a function of pH, the progressively greater loss of $^{14}CO_2$ as the pH was lowered from 7.0, could be taken into account. The results are presented in Fig. 2. Above pH 7.0, relatively little of the $H^{14}CO_3^-$ present becomes bound to the grana. When the pH is lowered from 7.0 to 5.4, about five times more of the $H^{14}CO_3^-$ present becomes bound.

Removal of tightly bound HCO_3^- and the effect on oxygen evolution

Chloroplasts were given $H^{14}CO_3^-$ under conditions allowing binding to take place, then washed twice to remove all trapped and loosely bound HCO_3^-. What remained was that taken up by the small high affinity pool. The chloroplasts were then washed a third time in various media and then collected by centrifugation. The amount of label remaining in the pellet was determined as well as the ferricyanide-supported oxygen evolving activity of the chloroplasts, first in the absence, then in the presence of 12 mM added $NaHCO_3$. Thus the effect of the third washing on both HCO_3^- removal and suppression of oxygen evolution could be observed.

The results are shown in Table II. As a control (line 1), the chloroplasts were washed a third time in the same solution as the first two washings (0.1 M sodium phosphate, pH 7.0, 0.01 M NaCl, 0.3 M sucrose). Afterwards, these chloroplasts retained a measurable amount of $H^{14}CO_3^-$ (4400 dpm/mg chlorophyll) and evolved oxygen normally in the absence of added HCO_3^-. When the third washing utilized high salt medium pH 5.0, i.e. "HCO_3^- depletion medium" used in the past to induce HCO_3^- dependence, the chloroplast pellet lost more than 99 % of its bound HCO_3^- (line 2). Any that remained could not be accurately determined above background. Oxygen evolution in these same chloroplasts was suppressed by more than 90 %. Adding back 12 mM HCO_3^- then increased oxygen evolution more than 10-fold.

When the chloroplasts were washed in medium at pH 5.0 but containing low salt concentration (line 3) only 11 % of the HCO_3^- was removed and little HCO_3^- dependence was observed on oxygen evolution. Similarly when the wash medium

TABLE II

EFFECTIVENESS OF VARIOUS WASH MEDIA AT REMOVING TIGHTLY BOUND $H^{14}CO_3^-$ AND INDUCING DEPENDENCE OF OXYGEN EVOLUTION ON ADDED HCO_3^-

The chloroplasts were charged with $H^{14}CO_3^-$ by suspension in 1 ml medium containing 0.1 M sodium phosphate, pH 5.8, 0.2 M NaCl, 168 μM $NaH^{14}CO_3$ (1.2 μCi) and 2 mg chlorophyll. After a 5 min incubation at 30 °C, 6 ml of ice-cold wash medium containing 0.1 M sodium phosphate, pH 7.0, 0.01 M NaCl, 0.3 M sucrose was added. After centrifugation the pellet was washed twice in 7 ml of the wash medium, then a third time in the medium indicated. The dpm were determined in the final pellet and portions were measured for oxygen evolving ability in saturating light immediately after suspension in reaction mixture containing 0.1 M sodium phosphate, pH 7, 0.175 M NaCl, 0.1 M sodium formate, 2 mM $K_3Fe(CN)_6$, 50 μg chlorophyll/ml. Initial rates were measured before and after injection of $NaHCO_3$ to 12. 5 mM. Where silicomolybdate (SiMo) and DCMU were both present in the wash solution, DCMU was added first.

Wash solution	dpm/mg chlorophyll $\times 10^{-1}$	HCO_3^- removed (% of control)	μmol O_2/mg chlorophyll per hr		
			$-HCO_3^-$	$+HCO_3^-$	$\frac{+HCO_3^-}{-HCO_3^-}$
(1) 0.1 M sodium phosphate, pH 7, 0.01 M NaCl 0.3 M sucrose	440	control	40.0	41.3	1.03
(2) 0.1 M sodium phosphate, pH 5, 0.175 M NaCl 0.1 M sodium formate	< 3	> 99	2.6	38.7	14.9
(3) 0.1 M sodium phosphate, pH 5, 0.01 M NaCl 0.3 M sucrose	392	11	38.7	49.0	1.27
(4) 0.1 M sodium phosphate, pH 7, 0.175 M NaCl 0.1 M sodium formate	305	31	17.2	33.0	1.92
(5) 0.1 M sodium phosphate, pH 6.5, 0.01 M NaCl 0.3 M sucrose, 0.02 M $NaHCO_3$	433	2	48.5	47.0	0.96
(6) 0.8 M Tris, pH 8 (30 min at 4 °C)	402	9	0.0	0.0	–
(7) 0.1 M sodium phosphate, pH 7.0, 0.01 M NaCl 0.3 M sucrose, 10^{-5} M DCMU	445	−1	13.2	12.0	0.91
(8) 0.1 M sodium phosphate, pH 7, 0.01 M NaCl 0.3 M sucrose, 0.2 mM SiMo	100	77	36.0	19.0	0.52
(9) 0.1 M sodium phosphate, pH 7, 0.01 M NaCl 0.3 M sucrose, 10^{-5} M DCMU, 0.2 mM SiMo	301	32	16.0	7.6	0.48

contained high salts but was also at a high pH (line 4) only about 30 % of the tightly bound HCO_3^- was removed and oxygen evolution was suppressed about 50 %. Clearly both low pH and high salt concentrations are needed for maximum removal of tightly bound HCO_3^- and also for suppression of oxygen evolution. High salt concentrations alone, i.e. at neutral pH, would, however, remove more bound HCO_3^- if the wash time was extended (data not shown).

When chloroplasts were washed in a low salt medium containing 20 mM of unlabelled $NaHCO_3$, all the labelled HCO_3^- was retained by the chloroplasts (line 5). This result indicates that HCO_3^-, once bound in the small high affinity pool, does not normally exchange with free HCO_3^- unless the bicarbonate is in the presence of high salt concentrations and low pH.

Chloroplasts washed with 0.8 M Tris buffer lose less than 9 % of their bound HCO_3^- (line 6) although oxygen evolution is completely suppressed as found by Yamashita and Butler [8]. In similar fashion chloroplasts washed with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) retain all their bound HCO_3^-. DCMU-washed chloroplasts do show some HCO_3^--insensitive oxygen evolution, since much of the DCMU was removed with pelleting and resuspension in DCMU-free reaction mixture. Clearly neither Tris nor DCMU act by removing bound HCO_3^- or by competing with HCO_3^- for binding sites.

A very curious result was obtained when the chloroplasts were washed with silico molybdate (SiMo). This substance was found by Girault and Galmiche [9] and by Giaquinta et al. [10] to permit oxygen evolution even in the presence of DCMU. At a concentration of 0.2 mM, SiMo removed 77 % of the tightly bound $H^{14}CO_3^-$ (line 8) while initial rates of oxygen evolution remained about the same as in the control. Once washed with SiMo, however, chloroplasts lose oxygen evolving activity very rapidly in light, as observed by Zilinskas and Govindjee [11] and this loss is not overcome by addition of HCO_3^-. The lower rates seen after addition of HCO_3^- reflect the rapid photoinactivation which took place during the first light period (measurement minus HCO_3^-) rather than what might appear to be HCO_3^- inhibition of oxygen evolution.

When DCMU is given to chloroplasts prior to washing with SiMo, less HCO_3^- is removed (line 9). Under the conditions of this experiment, SiMo removed 32 % of the tightly bound HCO_3^- when added after DCMU as opposed to 77 % when no DCMU was present. DCMU appeared to retard rather than prevent entirely the removal of HCO_3^- by SiMo. In both the presence and absence of DCMU, the removal of HCO_3^- by SiMo increases with concentration of SiMo, temperature and wash time (data omitted) but removal is always less in the presence of DCMU.

As a last treatment, chloroplasts having only their high affinity pool charged with $H^{14}CO_3^-$ were heated for 5 min at 45 °C. Such chloroplasts lost about 85 % of their bound HCO_3^- while oxygen evolution was completely destroyed (data not shown). Since heated chloroplasts still retained a measurable amount of bound HCO_3^- but lost all ability to evolve oxygen, heat damage probably involves more than destroying HCO_3^- binding sites although this is one definite consequence of the treatment.

Determining the small pool size

The fact that HCO_3^-, once bound in the small high affinity pool, cannot be

removed even with repeated washing in high pH, low salt, buffered solutions allowed a fairly precise determination of the small pool size. Chloroplast grana were first washed in depletion medium (0.1 M sodium phosphate, pH 5.0, 0.175 M NaCl, 0.1 M sodium formate) to remove all endogenous bound HCO_3^- (Table II, line 2). The chloroplasts were then given enough labelled HCO_3^- to refill the small pool of HCO_3^- binding sites completely as indicated by full restoration of oxygen evolving ability. The grana were then washed three or four times in medium containing 0.1 M sodium phosphate, pH 7.0, 0.01 M NaCl and 0.3 M sucrose to remove all trapped HCO_3^- while leaving the small pool of tightly bound HCO_3^- intact. After washing, the chlorophyll and HCO_3^- content of the chloroplast pellet was determined.

Maize chloroplasts treated in the above manner yielded values ranging from 2.77 to 2.92 nmol HCO_3^- bound per mg chlorophyll. The values were the same whether three or four washings were employed. Spinach chloroplasts, similarly treated, yielded values ranging from 2.61 to 2.96 nmol HCO_3^- bound per mg chlorophyll. Taking the molecular weight of chlorophyll to be 900, these numbers indicate that one HCO_3^- is bound per 380–400 chlorophyll molecules in maize with a very similar ratio in spinach chloroplasts. The number of Photosystem II reaction centers is usually taken as one per 300–400 chlorophyll molecules [12]. Thus one HCO_3^- is tightly bound for every Photosystem II reaction center.

The size of the small pool determined in the above fashion agrees fairly well with the preliminary estimation from the Scatchard plot (Fig. 1).

Effect of temperature on high affinity binding of $H^{14}CO_3^-$

Exchange of exogenous $H^{14}CO_3^-$ with previously bound HCO_3^- is temperature dependent. This conclusion is derived from the data presented in Table III. Chloroplasts were given $H^{14}CO_3^-$ under conditions (see legends, Table III) allowing some incorporation into the small pool. They were incubated 5 min at the indicated temperatures, then washed repeatedly to remove unbound $H^{14}CO_3^-$. Chloroplasts incubated at 30 °C were found to contain about 2.7 times more $H^{14}CO_3^-$ than those incubated at 1 °C.

TABLE III

BINDING OF EXOGENOUS HCO_3^- TO THE HIGH AFFINITY SITE AS A FUNCTION OF TEMPERATURE

Chloroplasts were suspended in 1 ml reaction mixture containing 0.1 M sodium phosphate, pH 6.5 (adjusted at the temperature of incubation), 0.2 M NaCl, 1.36 mM $NaH^{14}CO_3$ (0.5 μCi), 0.6 mg chlorophyll. Identical samples were incubated 5 min at the temperature indicated, then 6 ml of ice-cold wash medium containing 0.1 M sodium phosphate, pH 7.0, 0.01 M NaCl and 0.3 M sucrose was added to each. After pelleting and three washings, bound $H^{14}CO_3^-$ was measured.

Incubation temperature (°C)	HCO_3^- bound (nmol/mg chlorophyll)
1	0.50
15	0.87
30	1.35

Effect of light on tightly bound $H^{14}CO_3^-$

In all work mentioned so far the chloroplasts were kept in the dark throughout the experiment. Illuminating chloroplasts did not, in fact, produce any noticeable increase in $H^{14}CO_3^-$ uptake into either the large or small pool of binding sites. Thus uptake of HCO_3^- is a "dark" reaction. It was of interest to determine also if HCO_3^- may be released in light. Experiments to test this point produced largely negative results, at least with the small, high affinity pool.

Chloroplasts were charged with $H^{14}CO_3^-$ and then washed twice, leaving only the label incorporated in the small pool. They were then illuminated in saturating light for 18 min while oxygen evolution was monitored. Aliquots were drawn from the reaction mixture at intervals, centrifuged, and the amount of $H^{14}CO_3^-$ remaining in the pellet was determined. Results are depicted in Fig. 3. At the end of 18 min of illumination, net oxygen evolution in the chloroplasts was reduced to almost zero due to photoinactivation. Meanwhile the chloroplasts had lost progressively only about 35 % of the $H^{14}CO_3^-$ bound in the small pool. Dark controls, otherwise similarly treated, lost no $H^{14}CO_3^-$. The same results were obtained when 20 mM unlabelled $NaHCO_3^-$ was added to the reaction mixture. Again the chloroplasts evolved the same amount of oxygen and lost progressively only about 35 % of their bound $H^{14}CO_3^-$.

The latter results (with 20 mM added unlabelled HCO_3^-) indicate that while a small fraction of the tightly bound HCO_3^- is lost during prolonged illumination, there is no rapid exchange of the bound HCO_3^- with free HCO_3^- during oxygen evolution in the light. Rather, it appears that a HCO_3^- joins each of the high affinity binding sites, activates electron flow, and remains in place even in the presence of large amounts of free HCO_3^- while the reaction center evolves many molecules of oxygen.

The loss of bound HCO_3^- that does occur in the light may be the result of

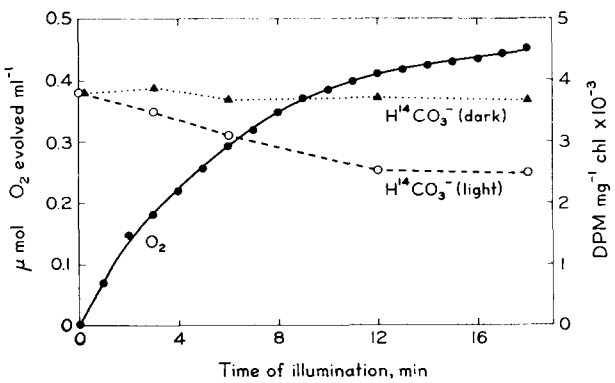

Fig. 3. Loss of tightly bound $H^{14}CO_3^-$ from chloroplasts during oxygen evolution. Chloroplasts were charged with $H^{14}CO_3^-$ as explained in the legend of Table II. After a third washing the grana were suspended in reaction mixture containing 0.1 M sodium phosphate, pH 7.0, 0.01 M NaCl, 0.3 M sucrose, 0.01 M $K_3Fe(CN)_6$, 162 μg chlorophyll/ml, then illuminated. At times indicated, 1 ml of the reaction suspension was withdrawn from the illumination chamber, centrifuged, and the pellet measured for bound HCO_3^-. Similar results were observed when 20 mM unlabelled $NaHCO_3$ was added to the reaction mixture before illumination.

membrane damage of a non-specific nature. There appears not to be any direct correlation between photoinactivation and destruction of high affinity binding sites. Chloroplasts having lost nearly all ability to evolve oxygen resulting from prolonged illumination still retain about 65 % of their tightly bound HCO_3^-.

DISCUSSION

While the data presented indicate the existence of two pools of binding sites for HCO_3^- in broken chloroplasts, the role each plays in stimulating Photosystem II is far from clear. Of the two, little has been said here regarding the large, low affinity pool. Preliminary experiments (data not presented) indicate that the large pool of binding sites may be progressively destroyed as chloroplasts become photoinactivated. Verification of this observation, however, as well as more complete characterization of the large pool, will be the subject of future study.

More can be said about the small, high affinity pool of HCO_3^- binding sites. Removal of this pool by washing with a high salt, low pH medium suppresses oxygen evolving ability more than 90 % (Table II). There are also ways of eliminating oxygen evolution which do not affect HCO_3^- binding sites, such as Tris washing. Thus Tris-washed chloroplasts would still be expected to show a bicarbonate effect using artificial electron donors to Photosystem II as was, in fact, shown by Wydrzynski and Govindjee [13].

Heat treatment, on the other hand, destroys HCO_3^- binding sites and heated chloroplasts do not show a bicarbonate effect with the artificial electron donor diphenylcarbazide [2]. Thus it would seem that the small pool of bound HCO_3^- does normally control the rate of electron flow through Photosystem II. The only apparent counter evidence is the curious ability of silicomolybdate wash to remove almost 80 % of the tightly bound HCO_3^- while leaving the initial rate of oxygen evolution nearly the same as unwashed controls. From the work of others [9–11, 14] it is obvious that the action of silicomolybdate is complex and imperfectly understood. The data presented here do not distinguish among the following possibilities. Silicomolybdate wash may allow more rapid electron flow through those 20 % of the reaction center complexes still associated with HCO_3^-. It may substitute functionally for HCO_3^- in the 80 % of the reaction center complexes from which HCO_3^- was removed, or it may permit ferricyanide to accept electrons before the HCO_3^- control point so that the destruction of HCO_3^- binding sites does not result in lower electron flow rates. No matter what silicomolybdate does, however, one could still argue that under "normal" conditions, HCO_3^- bound in the small pool does control electron flow rate.

The difficulty with which HCO_3^- is removed from the high affinity pool explains why chloroplasts normally show no dependence at all on exogenous HCO_3^- for O_2 evolution. Apparently chloroplast grana contain a full complement of bound HCO_3^- when isolated and do not lose this HCO_3^- as a consequence of the usual isolation procedures or even during prolonged Hill reaction studies. This pool of bound HCO_3^- can be exchanged or removed under certain conditions (low pH, high salt concentrations) but these two conditions are not usually met experimentally.

The location of the high affinity binding site can be deduced somewhat specifically from the evidence presented here. Since DCMU can, in the dark, severely retard

the removal of HCO_3^- by silicomolybdate it is reasonable to postulate a very close spatial relationship between the HCO_3^- binding site and the site of action of DCMU, though clearly the two substances do not compete for exactly the same binding site. It appears that DCMU "overlays" the bound HCO_3^-, protecting it from silicomolybdate attack as well as blocking electron flow. As it is believed that DCMU inhibits electron flow very near Q, the primary electron acceptor to Photosystem II, HCO_3^- either forms a part of Q, or forms part of the reaction center complex in the very immediate vicinity of Q. This is probably the site proposed by Wydrzynski and Govindjee [13] to exist based on fluorescence transients and the "major site" proposed by Jursinic et al. [15] based on the rate of reoxidation of Q^- and other evidence. Depleting chloroplasts of HCO_3^-, however, has two distinct effects. About half the Photosystem II reaction centers cannot evolve oxygen at all, while those still capable of evolving oxygen have markedly longer recovery times following a photoact [3]. The second effect is due to a slowing of electron transfer rates from reduced Q to secondary electron acceptors [15] and is almost certainly controlled by HCO_3^- bound into the small, high affinity pool of binding sites. Either this pool also controls the number of active reaction centers, or a second site of action of HCO_3^- must be proposed. If a second site of action does exist, to account for the number of reaction centers able to evolve oxygen, the large pool of low affinity HCO_3^- binding sites may be involved. This hypothesis is now being tested.

Aside from the location of the small HCO_3^- pool, there is the question of its overall function. Since HCO_3^- bound in this pool does not necessarily exchange with free HCO_3^- in either the dark or while oxygen is evolved in the light, it would appear that HCO_3^- has some sort of structural function. While it does not appear likely that this pool also has some catalytic chemical function, such a possibility cannot be excluded.

ACKNOWLEDGEMENT

I thank Dr. T. Wydrzynski for providing the sodium silico-12-molybdate.

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