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Bicarbonte stimulation of oxygen evolution, ferricyanide reduction and photoinactivation using isolated chloroplasts

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Dependence of Hill reaction (ferricyanide reduction) by isolated (broken) chloroplasts on bicarbonate ion increases with time of illumination (upto 4 min) in HCO_3^- free reaction mixture. The stimulation caused by HCO_3^- is independent of light intensity down to very low intensities indicating an involvement of this ion in early photochemical events of photosystem II. Oxygen evolution was found to be more dependent than ferricyanide reduction on HCO_3^-. The existence of an endogenous non-oxygen evolving electron donor in chloroplasts is thus suggested. HCO_3^- is also shown to greatly increase the rate of photoinactivation during Hill reaction.

The ability of the bicarbonate ion to stimulate Hill reaction in isolated chloroplasts is now well documented (*1–7*). HCO_3^-, rather than CO_2, appears to be the active moiety (*7*). There is now a substantial amount of evidence, including effects of HCO_3^- on chlorophyll *a* fluorescence and delayed light emission from chloroplasts, to indicate that HCO_3^- acts at, or near, the oxygen evolving steps (*7*, *8*).

The role of preillumination in increasing HCO_3^- dependence is a matter of controversy. Warburg and Krippahl (*1*), the discoverers of the HCO_3^- effect, claimed that preillumination was necessary to maximize HCO_3^- dependence. Good (*5*) preilluminated chloroplasts in the absence of ferricyanide and found no increased dependence on HCO_3^-. In this paper, we show that preillumination of chloroplasts in HCO_3^- free medium, but in the presence of ferricyanide leads to increased dependence of the reaction on HCO_3^-.

It is important to know whether or not HCO_3^- can stimulate Hill reaction at low light intensities (where the rate of the reaction increases linearly with increase in intensity) since a low intensity effect would indicate an involvement in photochemical reactions rather than in purely enzymatic ones. Unfortunately, the published data appear conflicting on this point (*5*, *6*). We, therefore, reinvestigated this problem and report here that the dependency of ferricyanide reduction on exogenous HCO_3^- is observed even at low light intensities, the HCO_3^- effect being independent of light intensities used.

Good (*5*) has reported observing an excess of 10 fold stimulation of oxygen evolution by HCO_3^- addition. However, those who have measured dichlorophenol

indophenol (DCPIP) or ferricyanide reduction failed to observe more than a 4–5 fold stimulation (*4*, *6*, *7*). To discover whether or not these differences are real we measured oxygen evolution and ferricyanide reduction in identical samples and found that oxygen evolution is indeed more dependent on HCO_3^- than ferricyanide reduction. To explain the differences, we suggest that some endogenous electron donor must exist which can donate electrons to photosystem II and reduce ferricyanide without evolving oxygen. In the presence of HCO_3^-, however, electron flow is "coupled" to oxygen evolution.

Materials and methods

1. *Chloroplast preparation and bicarbonate depletion*

Oat (*Avena sativa* var. Cleland) plants were grown and chloroplasts isolated from them in the same manner as maize (*7*). Chloroplasts were broken by subjecting them to an osmotic shock during isolation (*7*; also see below). Frozen chloroplasts were thawed before use in all experiments. In experiments in which material from oats was used, no attempt was made to deplete the chloroplasts of HCO_3^- prior to placing them on the platinum electrode.

Maize (*Zea mays*) chloroplasts were depleted of HCO_3^- as follows: they were placed in 0.25 M NaCl, 0.04 M Na acetate and sodium phosphate buffer pH 5.0, and then pure N_2 gas was bubbled through the suspension. We have made the point (*7*) that a low pH (about 5.0) is necessary for maximum HCO_3^- depletion. Further studies have shown, however, that not all chloroplasts can tolerate such a low pH. Chloroplasts from C_3 plants that possess the Calvin cycle pathway for CO_2 fixation (e.g., oat, spinach (*Spinacia oleracea*), romain lettuce (*Lactuca sativa*) and, to a lesser extent, pokeweed (*Phytolacca americana*)) precipitate at such low pH and are deposited on the sides of their container by the bubbling action of N_2 gas. Chloroplasts from C_4 plants that possess the Hatch-Slack pathway in addition to Calvin cycle pathway for CO_2 fixation (e.g., maize, foxtail (*Setaria viridis*), purslane (*Portulaca oleracea*) and *Sorghum bicolor*), on the other hand, show very little such tendency.[1] It is important to point out that whether or not chloroplasts can tolerate pH 5.0, or must be depleted of HCO_3^- at higher pH (5.8–6.0) to prevent precipitation, all material tested showed large (usually 4–10 fold) increases in either ferricyanide reduction or oxygen evolution when provided with HCO_3^-.

2. *Ferricyanide reduction: Amperometric method*

Initial rates of ferricyanide reduction were measured using a platinum rate electrode similar to that described by Joliot and Joliot (*9*) but used in the unmodulated mode. The platinum electrode was polarized at +0.7 volts relative to the Ag/AgCl electrode. Thus it measured ferrocyanide production.

Chloroplasts were placed on the platinum surface and covered with a dialysis

[1] It seems there may be some difference between these two groups of plants in the structural protein of their thylakoids causing one to precipitate under more acid conditions. Though this apparent difference in chloroplasts from C_3 and C_4 plants has not, to our knowledge, been previously reported, further verification and pursuit of the phenomenon was considered not within the scope of this investigation.

membrane. Through the chamber above this membrane flowed CO_2 free solution containing 0.25 M NaCl, 0.04 M Na acetate, 1 mM potassium ferricyanide and 0.05 M Na phosphate, pH 6.8. Flow through this chamber could be switched to permit entry of the same solution plus 0.01 M $NaHCO_3$. Above this chamber, separated by another membrane, was a second chamber housing the Ag/AgCl electrode. Through this chamber flowed solution free of Hill oxidant.

The sample was illuminated with white light (approximately 10^6 ergs/cm^2·sec) from a tungsten ribbon filament lamp. The beam passed through a heat filter, focusing lens, shutter, and buffer (path length, 2 cm) before striking the sample. Assays were conducted using 0.5 sec shutter openings. The signal generated at the electrode was amplified by a Keithley microvolt ammeter (model 150B) and recorded, on a Heath Servo recorder (model EUW-20A) as a "spike" on the chart paper. At low light intensity the spike height was linear with intensity, allowing a good means of measuring relative quantum yields of ferricyanide reduction. Light intensity was reduced from saturation by means of neutral density filters.

After placing the chloroplasts on the electrode, several minutes were allowed for settling, after which the ability to reduce ferricyanide was assayed in the absence of exogenous HCO_3^- by means of several half-second flashes of light given at 30 sec intervals. The flow of buffer over the chloroplasts was then switched to that containing HCO_3^- and several more half-second assays were conducted. The effect of pretreatments in enhancing the stimulation seen when HCO_3^- was added was observed.

3. *Ferricyanide reduction: Spectral method*

Ferricyanide reduction was measured as an absorbance change at 420 nm using a Cary 14 recording spectrophotometer equipped with a side attachment to illuminate the sample. For both oxygen evolution (see below) and ferricyanide reducing measurements, actinic illumination came from GE 120V, 650W, DVY lamps. The beams passed through a 15 cm thick layer of water and Corning C.S. 3–71 yellow cut-off filters before emerging with intensities of 5×10^5 ergs/cm^2·sec. When simultaneous measurements of oxygen evolution and ferricyanide reduction were made, chloroplasts were suspended in reaction mixture and then aliquots were drawn off and placed in the respective instruments. Thus oxygen evolution and ferricyanide reduction could be compared using identical samples.

4. *Oxygen evolution*

Net oxygen evolution was measured by a Clark-type electrode set up using a Yellow Springs Oxygen Monitor (model 53). The signal was recorded by an Esterline Angus (model E11015) recorder.

Results and discussion

1. *Increase in bicarbonate dependence with preillumination*

Oat chloroplasts not previously depleted of HCO_3^- were placed on the platinum electrode. They were permitted to perform Hill reaction i.e., they were illuminated, in the presence of ferricyanide, for a variable length of time while HCO_3^- free solution passed over the membrane holding them to the surface of the platinum,

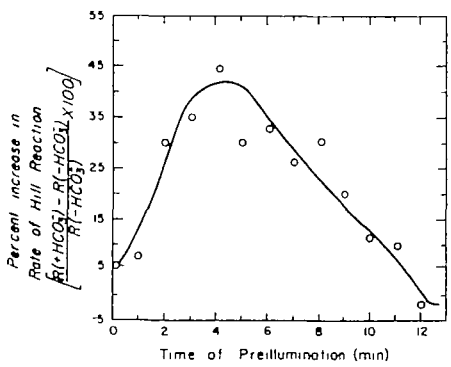

Fig. 1.

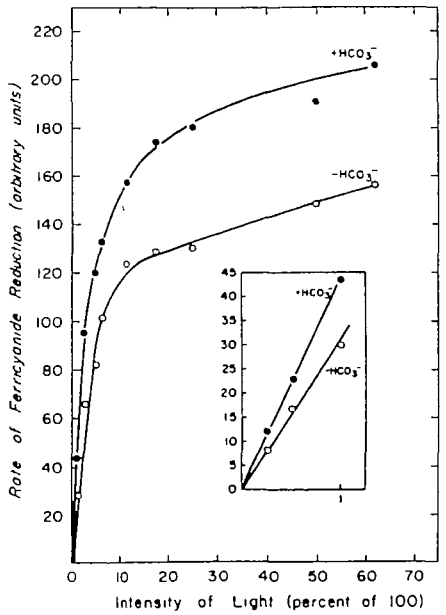

Fig. 2.

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 Na phosphate, pH 6.8, 0.5 mM potassium ferricyanide $\pm$0.01 M $NaHCO_3$. Saturating white light (10^6 ergs/cm^2-sec) used during preillumination and half-second assays. Anaerobic conditions. Oat chloroplasts (43 μg chlorophyll/ml in stock). Average of two series.

Fig. 2. *Rate of ferricyanide reduction with and without 0.01 M $NaHCO_3$ as a function of light intensity.* Oat chloroplasts preilluminated 4 min in saturating white light. Light intensity was varied with calibrated neutral density filters. Average of 3 series. Other conditions as in Fig. 1.

After this period of illumination, several half-second assays were conducted. The chloroplasts were then given HCO_3^- containing solution and several more half-second assays were performed.

Dependence of Hill reaction on HCO_3^- as a function of preillumination time is shown in Fig. 1. The first 4 min of preillumination cause a progressive increase in HCO_3^- dependence. After this time, dependence again decreases to essentially 0 between 10 and 12 min. Dark controls showed no change in dependence on HCO_3^-. Likewise illuminating chloroplasts in the absence of ferricyanide did nothing to increase the dependence of Hill reaction on HCO_3^-. In this respect we confirm the results of Good (*5*) who also showed that preilluminating chloroplasts in the absence of ferricyanide did not lead to greater HCO_3^- dependence of the Hill reaction. Good, however, did not attempt to observe increased HCO_3^- dependence with preillumination in the presence of ferricyanide. It appears, therefore, that increased HCO_3^- dependence of the Hill reaction is a function of electron flow rather than light absorption alone. It should be pointed out that the decline in HCO_3^- dependence after 4 min is associated with a decline in the over-all activity, both of which are reduced to nearly zero after 10–12 min.

It should also be mentioned that the results presented in Fig. 1 were most reproducible when chloroplasts were frozen for at least a week. Fresh material, or that frozen less than about 1 week, also showed increasing dependence of Hill reaction on exogenous HCO_3^- for the first 4 min of preillumination. A difference using fresh material was seen after 4 min. In this case both over-all activity and HCO_3^- dependence were maintained for much longer, declining to zero only after about 20 min. Prolonged freezing may be causing a slow destruction of membrane integrity accounting for the more rapid loss of activity and associated decrease in HCO_3^- sensitivity.

Since HCO_3^- is not "used up" during Hill reaction (which would imply permanent incorporation[2] into an organic molecule) increasing HCO_3^- dependence during Hill reaction must result in some other way. We therefore suggest, as in an earlier publication (*7*), that HCO_3^- is initially bound, perhaps directly to reaction centers, or exists in some complexed condition. As Hill reaction proceeds, it becomes unbound, or otherwise free (as CO_2?). This "free" HCO_3^- may be recycled to some extent within the thylakoids, but increasing dependence of the reaction on exogenous HCO_3^- implies that at least some is lost. It is also clear from the fact that HCO_3^- dependence decreases after 4 min of illumination that at least one other factor limits the reaction. This factor, usually described by the general term of photoinactivation, is not well understood (*10–13*).

2. *Bicarbonate effect as a function of light intensity*

Oat chloroplasts, again not previously depleted of HCO_3^-, were placed on the platinum electrode and allowed to perform Hill reaction in the absence of exogenous HCO_3^- for 4 min. This induced 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 using calibrated neutral density filters. Fig. 2 shows that the bicarbonate effect seen under these experimental conditions is independent of light intensity. Even at that lowest intensity (in the linear portion of the light curve) where ferricyanide reduction could still be accurately measured (see insert), HCO_3^- stimulated the activity to the same degree as at saturating intensity. This is shown by the constancy of the ratio of the rate of Hill reaction in the present to that in the absence of HCO_3^- as a function of light intensity. These results differ with those published by Good (*5*) who measured oxygen evolution manometrically and showed less stimulation of HCO_3^- at low intensity than at high. However, West and Hill (*6*), measuring DCPIP and ferricyanide reduction spectrophotometrically, showed that with DCPIP reduction the HCO_3^- effect was independent of light intensity but with ferricyanide reduction, dependence on HCO_3^- was less at low intensity. We feel that our results are more reliable for several reasons: a) since assays consisted of half-second flashes of actinic light, the data more aptly reflect the ability of HCO_3^- to stimulate initial rates of ferricyanide reduction at the various intensities; b) the same sample was used without, and then with HCO_3^-, at each intensity; and c) the method of assay used in our work here, is more sensitive and accurate, especially at low intensities than spectrophotometric measurements since ferricyanide

[2] A. Stemler (unpublished) found no permanent incorporation of $H^{14}CO_3^-$ into organic molecules; Also see reference *26*.

has a fairly low absorbtivity even at the 420 nm wavelength where it is usually measured.

We conclude from the results in Fig. 2 that HCO_3^- is involved in early "photochemical reactions" of photosystem II rather than enzymatic reactions somewhat removed from the reaction centers.

3. *Comparison of the effect of bicarbonate on oxygen evolution and ferricyanide reduction*

When maize chloroplasts are depleted of HCO_3^-, over 90% of their oxygen evolving ability is suppressed. However their ability to reduce ferricyanide is suppressed by less than 80%. This is shown in Fig. 3. HCO_3^- can increase ferricyanide reduction 4–5 fold whereas oxygen evolution, in the same samples, is increased over 15 fold. In the presence of HCO_3^-, in contrast, equal μ equivalents of oxygen and ferricyanide are produced, at least during the first several minutes of illumination. That is, in the presence of HCO_3^-, for every molecule of oxygen evolving, 4 electrons are transferred to ferricyanide.

To explain the differences in O_2 evolution and ferricyanide reduction, we may suggest that there exists a fairly substantial amount of an endogenous electron donor capable, under certain circumstances, of donating electrons to photosystem II without evolving oxygen. This will account for the greater equivalents of ferricyanide reduced than oxygen evolved from HCO_3^- depleted chloroplasts. This interpretation is consistent with other available data. Residual ferricyanide reduction in the absence of oxygen evolution was first observed by Kahn (*14*),

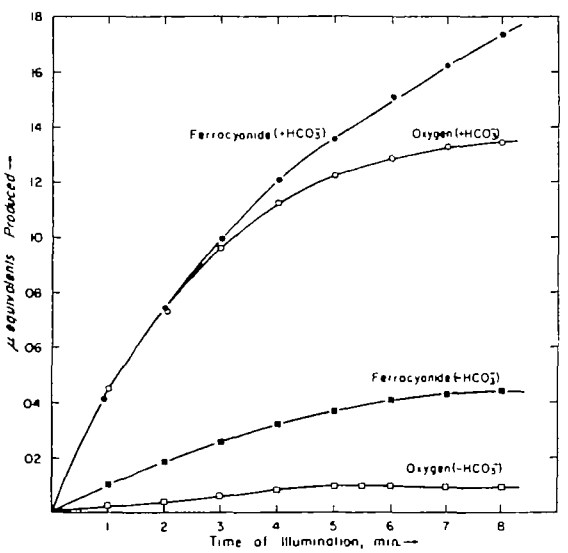

Fig. 3. *Comparison of oxygen evolution and ferricyanide reduction in the presence and absence of* HCO_3^-. Reaction mixtures contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate, pH 4.8, 1 mM potassium ferricyanide $\pm$ 0.01 M $NaHCO_3$ and 15 μg of chlorophyll/ml of maize chloroplast suspension. The light intensity was 5×10^5 ergs/cm^2-sec. Corning C.S. 3-71 (yellow) cut-off filter was used. Initially, anaerobic conditions.

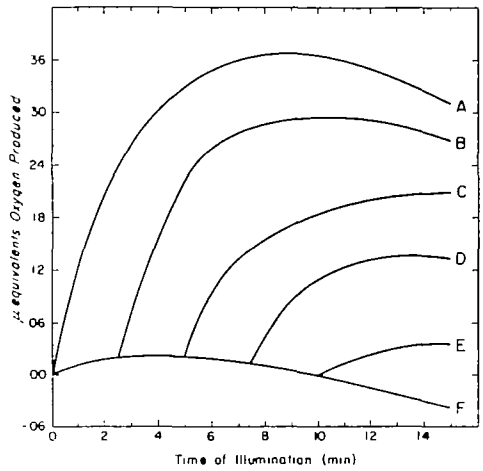

Fig. 4. *Oxygen evolution in response to (10 mM) HCO_3^- injected into different samples after varying periods of illumination.* The reaction mixture contained 0.25 M NaCl, 0.04 M Na acetate, 0.05 M phosphate, pH 6.8, 2 mM potassium ferricyanide and 40 μg of chlorophyll/ml of maize chloroplast suspension. Curves A, B, C, D and E represent oxygen generated after injection of HCO_3^- to 0.01 M at 0, 2.5, 5, 7.5 and 10 min respectively. Chloroplasts not given HCO_3^- at all generated oxygen as represented by curve F. The 2 min dark interval, required to achieve a flat baseline after injection of HCO_3^- solution, was omitted in redrawing the traces. Other conditions were the same as in Fig. 3.

using a protein-chlorophyll complex isolated from chloroplasts after treatment with Triton X-100. Kutyurin et al. (*15*), using chloroplasts and measuring oxygen evolution and ferricyanide reduction simultaneously, found that oxygen evolution was more inhibited by treatment with low concentrations of dodecylsulfate, digitonin and CMU. Most recently, Huzisige and Yamamoto (*16*), using photosystem II particles, found residual ferricyanide reduction without oxygen evolution. These last authors showed that the ferricyanide reduction was not simply an apparent absorbance change caused by pigment photobleaching. No significant absorbance change was seen when ferricyanide was omitted from the reaction mixture during illumination. (Our control experiments gave similar results.) In addition these authors used colorimetric methods to confirm that ferricyanide was, in fact, reduced under conditions where no oxygen was evolved.

In light of the work of Yamashita et al. (*17*), however, the question of pigment photobleaching may still be raised. These authors have shown that carotenoids are irreversibly photobleached when chloroplasts are illuminated in the presence of ferricyanide and certain inhibitors of the Hill reaction but not in the absence of these substances. The absorbance change at 420 nm due to photobleaching is small (approximately 25 percent) relative to the change seen at 500 nm. In our control experiments we also see a small change in absorbance at 500 nm during illumination indicating some pigment bleaching. However, the amount of change at 500 nm is always less than 30 percent of the absorbance change at 420 nm. We therefore estimate that pigment photobleaching can account for only a portion of the absorbance change we observe at 420 nm. The remaining change must be due to ferricyanide reduction.

Despite considerable evidence of its existence, the identity and the exact amount of this endogenous electron donor is unknown. It is difficult even to imagine what could exist in such large amounts within the thylakoid membrane or photosystem II particles. Still another interesting consideration is the possible role of this donor in the functioning of photosystem II and also its relationship to HCO_3^-. Our data in Fig. 3 can be explained by postulating that HCO_3^- stops the "abnormal" flow of electrons from this endogenous donor, switching the flow so that electrons come from the oxygen evolving system.[3] Another possibility is that HCO_3^- and this unidentified donor actually cooperate in some fashion to evolve oxygen. Thus both may be necessary for oxygen evolution. One may speculate that photoinactivation could be due at least in part to the depletion of this required substance which can, under certain circumstances such as HCO_3^- depletion, act as an electron donor. This idea will be later substantiated (see discussion of Fig. 5).

4. *The effect of bicarbonate injected after different periods of illumination*

Maize chloroplasts were depleted of HCO_3^- and illuminated in the presence of ferricyanide. After different periods of time, illumination was stopped, HCO_3^- was injected and illumination resumed after 2 min. The initial rates of oxygen

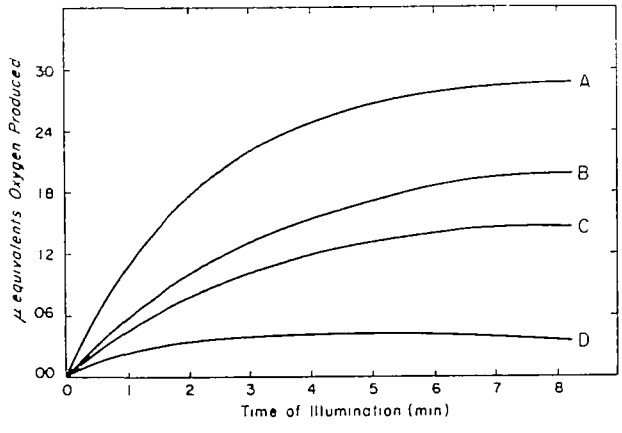

Fig. 5. *Residual oxygen evolution after 8 min of pretreatment.* During the assays shown here all samples contained the same reaction mixture as in Fig. 4. Illumination conditions were the same as in Fig. 3. Initially, anaerobic conditions. The pretreatments were: (A): 8 min dark, + ferricyanide ±10 mM HCO_3^-; (B): 8 min saturating light, −ferricyanide±HCO_3^-; (C): 8 min saturating light, + ferricyanide, −HCO_3^-; (D): 8 min saturating light, + ferricyanide, +HCO_3^-.

[3] A differential effect of HCO_3^- on ferricyanide reduction and oxygen evolution is further evidence that this ion acts on the oxygen evolving side of photosystem II. If HCO_3^- ions were acting between Q and A, it is difficult to see how a differential effect would be obtained (see Fig. 3, this paper). However, if HCO_3^- ions were acting on the "S" states leading to oxygen evolvion, then ferricyanide reduction, taking place at the expense of an endogenous, non-oxygen evolving, donor that donates electrons directly to the oxidized reaction center chlorophyll, might be less sensitive to HCO_3^- and a differential effect on ferricyanide reduction and O_2 evolution could be explained.

evolution and the net amounts of oxygen evolved are shown in Fig. 4. Curve F resulted from illuminating chloroplasts for 15 min in the absence of HCO_3^-. Curves A, B, C, D and E were obtained by injecting HCO_3^- at 0, 2.5, 5, 7.5 and 10 min into different samples respectively. (The 2 min dark interval, required to achieve a flat baseline after injection of the HCO_3^- solution, was omitted in redrawing the traces.)

We can see from this family of curves (A–E) that as HCO_3^- injection is delayed, the net amount of oxygen evolved and the initial rate of evolution after injection, declines. For example, if HCO_3^- injection is delayed 7.5 min, both the initial rate of oxygen evolution, and the maximum amount of oxygen evolved, are reduced about 60%. Thus it is clear that photoinactivation is taking place in the absence of HCO_3^- and in the absence of substantial amounts of net oxygen evolution. Further data on photoinactivation including dark controls will be discussed below.

5. *The effect of bicarbonate on photoinactivation of chloroplasts*

HCO_3^- depleted chloroplasts were illuminated with saturating light for 8 min under various conditions. Residual oxygen evolving ability was then assayed during a second 8 min period. During the assay period both ferricyanide and HCO_3^- were present in all samples. The results are shown in Fig. 5. Curve A shows the remaining activity in the dark control. These chloroplasts were kept 8 min in the dark in the presence of ferricyanide and either with or without HCO_3^-. Curve D shows the residual activity in chloroplasts which were illuminated 8 min in the presence of both ferricyanide and HCO_3^-. After this treatment, the oxygen evolving activity is reduced by 85%. Curve C shows the activity remaining after 8 min of illumination in the presence of ferricyanide but in the absence of HCO_3^-. In this case activity was reduced 50%. Curve B was obtained using chloroplasts illuminated 8 min in the absence of ferricyanide and either with or without HCO_3^-. These treatments reduced activity only about 30%.

We can draw several conclusions from these data concerning both the HCO_3^- effect and photoinactivation. Clearly HCO_3^-, which markedly increases electron flow coupled to oxygen evolution, is also very effective in accelerating photoinactivation (Curve D). Illumination in the presence of ferricyanide alone ($-HCO_3^-$) also leads to somewhat less photoinactivation through very little oxygen is evolved (see Fig. 3 and 4). It should be kept in mind that electron flow is taking place under this condition even if it is not necessarily coupled to oxygen evolution (Fig. 3). Photoinactivation then, under these experimental conditions, seems more a function of the amount of electron flow (from whatever source) than the amount of oxygen evolved.

Since all of the above-mentioned treatments were done under initially near-anaerobic conditions, we thought it might be possible that the increased photoinactivation seen in the presence of HCO_3^- might be due to an indirect effect of the increased amount of oxygen which develops under this condition (see Fig. 3 and 4). We, however, found that increasing the oxygen concentration in samples illuminated in the absence of HCO_3^- did not appreciably increase photoinactivation.

Samples which were illuminated in the absence of ferricyanide also showed some photoinactivation (Curve B, Fig. 5). It cannot be argued, however, that this amount of photoinactivation is due to some direct irreversible damage by

light rather than an effect of electron flow. Such direct damage is associated with very high light intensities (*18*). Moreover it is known that a considerable amount of oxygen uptake can be measured when chloroplasts are illuminated in the absence of a Hill oxidant (*19–21*). We also monitored oxygen during illumination of chloroplasts in the absence of ferricyanide and observed net oxygen uptake, even in samples made anaerobic before illumination. This net oxygen uptake of the trace amounts of oxygen remaining is about the same in the presence or absence of HCO_3^-. It is probable then that at least some, and perhaps all, of the "photoinactivation" reported here in the absence of a Hill oxidant can still be correlated to electron flow rather than to some direct damage by light.

Photoinactivation has been shown by Satoh (*10, 13*) to be a complex phenomenon, different for photosystems I and II. The usual way of explaining photoinactivation of photosystem II is to assume the accumulation of reduced intermediates which react with, and damage, the system itself. If this were the case, one might expect that providing an electron sink such as ferricyanide, would prevent the accumulation of these reduced intermediates and hence retard photoinactivation. (Ferrocyanide itself, rather than damage chloroplasts, has been shown by Brewer and Jagendorf (*22*) to have a protecting effect.) This is not the case. Greater damage is seen in the presence of ferricyanide. We, therefore, suggest that photoinactivation of photosystem II may, in part, be due rather to the depletion of a pool of some substance required for the functioning of the oxygen evolving side of the photosystem. This substance may be the same one which appears as a non-oxygen evolving electron donor in the absence of HCO_3^-. This hypothesis also implies that this unidentified material is used up even more rapidly in the presence of HCO_3^- since this ion accelerates photoinactivation.

Concluding remarks

There are a number of possible roles for HCO_3^- in the oxygen evolution process. Warburg and Krippahl (*1*), the discoverers of the HCO_3^- effect, offered the phenomenon as proof of Warburg's theory that oxygen evolves from the splitting of CO_2, and not from the splitting of H_2O. Metzner (*23*) has published a scheme in which HCO_3^-, bound to an acceptor, acts as the immediate electron donor to photosystem II. HCO_3^- may be simply an allosteric effector of the oxygen evolving enzyme or it may modify membrane potential. Stemler and Govindjee (*7, 8*) have proposed that HCO_3^- allows the formation of higher oxidation states of Z, the electron donor of photosystem II, in reference to the kinetic model of Forbush et al. (*24*). We now have some evidence for this effect on Z (Stemler et al., *25*). However, since none of these possibilities have been completely eliminated, nor are they all mutually exclusive, we are continuing the investigation of this phenomenon.

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