Bicarbonate Stimulation of Electron Flow in Thylakoids

Doug Jordan* and Govindjee†

Departments of Physiology & Biophysics,† Botany† and Agronomy,*
University of Illinois, Urbana, IL. 61801 (USA)

Offprint from

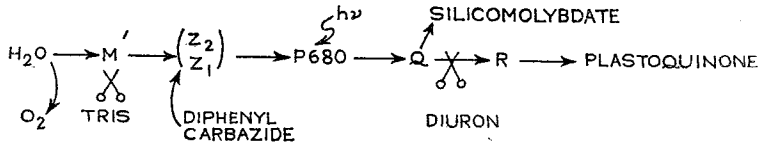
National Academy of Sciences, India : Golden Jubilee Commemoration Volume, 1980

In addition to being the source of fixed carbon in photosynthetic carbon assimilation, bicarbonate* serves as a reversible *in vitro* activator of three major photosynthetic processes : (I) The carbon dioxide fixing enzyme, RUBP carboxylase, is known to be activated by bicarbonate[12, 13, 15]. The level of bicarbonate required for half maximal activity has been shown to depend on the concentration of the essential metal activator, the pH, and the presence of a number of organophosphate activators. Though bicarbonate has been treated as an essential activator, the absolute requirement for bicarbonate has not been clearly documented. (II) Photophosphorylation rates and the P/e ratio, as studied in broken chloroplasts, are stimulated by the addition of bicarbonate[1, 2, 16, 17]. Bicarbonate enhancement of phosphorylation was most pronounced in a photosystem II** (PS II) independent cyclic phosphorylation system, as induced by pyocyanin. In a similar system, bicarbonate was shown to increase the levels of "energetic intermediates" of ATP synthesis[1, 2]. These effects have been noted to have a seasonal dependence with respect to the source of chloroplasts, and this is thought to be related to the hormonal status of the plant material[1]. A seasonally independent bicarbonate effect has been demonstrated with purified coupling factor I[14], in which bicarbonate stimulates the rates of magnesium and calcium ATPase activities. Bicarbonate is a nonessential activator in these systems since complete removal of bicarbonate does not eliminate phosphorylation ability. In addition, several carboxylic acids are nearly as effective as bicarbonate in stimulating coupling factor I activities[14], so the bicarbonate effect may be more appropriately categorized as a nonspecific

---

* Except where the question of the activating species is addressed, the word bicarbonate is used without implying that it is the activating species.

** Photosystem II of green plants consists of the following components : a light-harvesting complex that feeds energy to a reaction center chlorophyll *a* labelled as P 680 ; an electron acceptor quinone molecule labelled as Q ; a 2-electron acceptor quinone molecule, labelled as B or R, that accepts electrons from Q; two electron donors[7] Z_1 and Z_2 that donate electrons to $P\,680^+$; and an oxygen evolving Mn-containing enzyme (M) that receives electrons from H_2O. The sequence is written as :



activation by carboxylic acids. (III) Bicarbonate activation of the Hill reaction (electron flow from H_2O to an artificial electron acceptor) was the first of these bicarbonate activated systems to be discovered[26, 27, 28] and in recent years a considerable amount of scientific effort has been devoted to understanding this phenomenon. In comparison to photophosphorylation, the Hill reaction is activated by much lower concentrations of bicarbonate, and the requirement for bicarbonate appears to be both specific and absolute. Through the use of artificial electron donors and acceptors combined with several physical methods, the sites of bicarbonate activation in PS II electron transport and on the PS II reaction center have been systematically elucidated. The following paragraphs are devoted to reviewing the experimental evidence* characterizing the effects of bicarbonate on PS II activity and defining some of the questions that await experimentally determined solutions. The terminology used throughout the text is based on a recent review of this topic[5]. The term *thylakoid* refers to membranes isolated from chloroplasts.

The essentiality of bicarbonate for Hill reaction activity may be inferred from studies which show that bicarbonate depletion results in a 90-95% loss in Hill reaction rates[4,6,8,10,19-23,30]. The results of recent studies[11,20] indicate that the residual activity is probably due to incomplete removal of bound bicarbonate. The existence of high affinity and low affinity bicarbonate binding sites in chloroplast fragments was demonstrated. The removal of tightly bound bicarbonate was shown to be a slow process when chloroplast fragments were incubated under low salt, and neutral pH conditions. Removal of labeled bicarbonate was found to be facilitated by washing the chloroplasts with a low pH buffer containing high levels of sodium chloride and sodium formate, which had been found to be effective in depleting chloroplasts of Hill reaction activity in earlier bicarbonate activation studies[3,4,8,10,19-23,30]. By washing bicarbonate labeled chloroplasts with neutral pH, low salt buffer, the high affinity bicarbonate sites could be selectively preloaded with labeled [^{14}C] bicarbonate. Chloroplasts were prepared in this fashion and then various treatment washes were applied to samples of the labeled preparation. The treatments removed bicarbonate to varying degrees and Hill reaction activity was found to correlate well with the amount of label which remained bound. These results are important for two reasons.

* This review includes papers published up to 1979.

First, the correlation of bound bicarbonate with Hill activity provides firm physical evidence for bicarbonate activation. Second, the demonstrated effectiveness of the high salt and low pH wash medium in removing the tightly bound bicarbonate activator confirms that the effectiveness of this treatment is related to its ability to remove the effector and not to some sort of ion inhibition.

A previous study had shown that the requirement for salt treatment of chloroplasts was restricted to the bicarbonate depletion medium, and that bicarbonate sensitivity was observable in reaction media with or without high levels of salt[21]. It was also known that the effect of 100 mM formate in the incubation mixture increased the concentration of bicarbonate needed for half saturation of Hill activity by an order of magnitude[10], suggesting a competition by formate for bicarbonate binding sites.

The tight binding and slow exchange of bicarbonate at the activating sites explains the variability of early demonstrations of bicarbonate enhancements. Warburg[26,27,28] and several other researchers[3,24,25,29] had found it necessary to incubate chloroplast preparations for two or three hours, in the presence of bicarbonate traps, in order to observe bicarbonate enhancements of Hill reaction activity. These long-term incubations (some of which were conducted in the light) produced irreversible inactivation of the chloroplasts and probably were not totally effective in removing the activating species under investigation. So, the early reports are only useful for qualitative evaluations.

The universality of the bicarbonate effect can be inferred from the studies by independent laboratories, which used different higher plant and algal chloroplast material in their investigations. This point was emphasized by Stern and Venessland[25] who showed that the degree of bicarbonate enhanced Hill activity was the same in several different species.

In order to address criticisms that suggested that the bicarbonate effects were due to photodestruction of chloroplast activity during prolonged treatments, Good[3] showed that added bicarbonate was effective in restoring Hill activity to bicarbonate depleted chloroplasts, which had been pretreated with or without light. More recent studies have demonstrated the reversibility of each of the observed effects of bicarbonate. Good[3] and Stemler and Govindjee[21] noted that bicarbonate was more effective if the chloroplasts were incubated in the dark prior to measuring Hill reaction activity. Stemler reports that this is due to the preferred binding of bicarbonate to chloroplast

having the two electron acceptor quinone, R, in the oxidized state (as it would be in the dark).

The specificity for bicarbonate appears to be absolute. A number of carboxylic acids and other anions have been shown to be ineffective in raising Hill reaction activity in the absence of bicarbonate[3]. Additionally, bicarbonate has been shown to be a poor effector of the chloride activator, which has been shown to be located on the oxidizing side of PS II[9]. As mentioned earlier, bicarbonate activation of the Hill reaction has been differentiated from the bicarbonate activation of photophosphorylation. The lack of specificity for the activator of photophosphorylation is one major difference. In addition, the Hill reaction bicarbonate enhancement is more pronounced in uncoupled chloroplasts[3,10,29] ; while, bicarbonate stimulation of photophosphorylation is greater in a PS I cyclic phosphorylation system.

The bicarbonate effect on the Hill reaction was obverved to be more pronounced at high light intensities by most investigators[3,6,19,23,29]. The effect at low light intensities was less defined and some of the experimental results are still unexplainable. For example. West and Hill[29] found that a small bicarbonate effect was observed at low light intensities when DCPIP (2, 6 dichlorophenol indophenol) was the Hill oxidant, but no effect of bicarbonate was observed using ferricyanide. Stemler and Govindjee[22] observed the same bicarbonate dependence at low light intensities as at high light intensities when the ferricyanide Hill reaction was measured amperometrically, but when the same reaction was measured polarographically a definite light dependency was evident[23]. The larger bicarbonate effect at high light intensities indicated a bicarbonate requirement for a dark reaction of PS II. Further definition of this effect and convincing evidence for an effect on PS II chemistry was provided by measuring partial Hill reactions and transient intermediates using chemical and physical methods[8,10].

There are three lines of evidence supporting a bicarbonate enhancement of PS II chemistry. First, when oxygen flash yields were measured following widely spaced (1 s) saturating light flashes, bicarbonate depleted chloroplasts yields were approximately half those of control and bicarbonate replenished chloroplasts[23]. This approximated the bicarbonate enhancement of the DCPIP Hill reaction at low light intensities in the same study. Second, Jursinic *et al.*[8], measuring the electron spin resonance signal II_{vf}, corresponding to oxidized Z_2, found that the signal was depressed by 40% in bicarbonate depleted samples. Since the μs rise kinetics in chlorophyll a fluorescence

was shown to be the same in chloroplasts with or without bicarbonate, the rate constant of electron flow from Z to P-680 was taken to be independent of bicarbonate and the ESR signal depression could be assigned to a difference in PS II reaction center activity. Third, the levels of reduced quinones (mainly Q and R at 334 nm) were measured by absorption spectroscopy following a rapid succession of saturating light flashes[19]. Bicarbonate depletion resulted in an initial absorption decrease of 40%, indicating that the PS II reaction centers were inhibited.

The bicarbonate effect on the dark reactions was originally thought to involve the oxidizing side of PS II[21,22,23]. When chloroplasts were heat treated in order to inactivate oxygen evolution capability, bicarbonate produced no effect on the partial Hill reaction going from DPC (diphenyl carbazide) to DCPIP. DPC donates electrons to Z_1, so bicarbonate was thought to activate between the oxygen evolving site and Z_1. A later study by Wydrzynski and Govindjee[30] demonstrated that when alkaline Tris washing was used to inactivate the oxygen evolving system, the rate of DCPIP reduction, using DPC as the donor, was increased by more than twofold when bicarbonate was present. Control chloroplasts, not treated with Tris, showed a tenfold bicarbonate enhancement of DCPIP reduction. The discrepancy in the two studies may be explained by the report[20] that heat treatments irreversibly inactivate the bicarbonate activating site, whereas Tris treatment does not. There is an, as yet, unexplained activation of DCPIP reduction by the electron donor DPC, which must account for the reduced amount of bicarbonate activation seen when it is used.

Khanna *et al.*[10] established the assignment of a major bicarbonate dark effect to the reducing side of PS II by showing that the partial Hill reaction from water to silicomolybdate was not affected by bicarbonate. (Silicomolybdate accepts electrons from Q.) Electron flow from water to DAD (diaminodurene) was limited by bicarbonate depletion (to the same extent as ferricyanide reduction) and it was concluded that electron flow between Q and PQ (plastoquinone pool) was retarded.

Further evidence for a bicarbonate effect past Q was shown by variable chlorophyll a fluorescence measurements. The chlorophyll a florescence transient was shown to be dramatically altered by bicarbonate (reviewed in ref. 5). Bicarbonate depleted chloroplasts exhibit an initial rapid rise in chlorophyll a fluorescence ($O \rightarrow I$) followed by a slow second phase ($I \rightarrow P$); the first phase gives the appearance of a process as is seen with diuron

poisoning. (Diuron blocks electron flow from Q- to R). Bicarbonate restored the normal biphasic florescence pattern by slowing the initial phase and accelerating the second phase. (For further explanation, see ref. 5.) Wydrzynski and Govindjee[30] showed that Tris treated chloroplasts, supplied with a variety of electron donors, still required bicarbonate in order to restore a normal biphasic florescence transition pattern, thus indicating a bicarbonate requirement on the reducing side of PS II.

The dark decay kinetics of the ESR signal II_{vf} measuring oxidized Z_2, was found to be unchanged by the presence of bicarbonate, indicating that the rate constant of the 10 ns electron flow was not limited from H_2O to Z_2 and that the bolck was on the reducing side of PS II[8].

Chlorophyll a fluorescence yield decay measurements were made as another test for a block after Q. This method followed the fluorescence yield of chloroplast preparations (using a weak nondisturbing light source) after treatment with a short (10 ns), saturating light flash. Bicarbonate depleted samples were found to have fluorescence yield decay half-lives of about 3 ms, while bicarbonate sufficient chloroplast half-lives were about 0.5 ms[8]. Siggel *et al.*[19], using absorption spectrescopy, found that the half life for the reoxidation of Q^- in bicarbonate depleted chloroplasts was extended to about 10 ms from the 0.5 ms half-life observed in bicarbonate sufficient samples. The observed retardation of Q^- oxidation matches the measured extension in S_2 state relaxation times ($S'_2 \rightarrow S_3$) determined by measuring oxygen yield following three light flashes with variable spacing between the first and second flashes[23]. (The S states represent the status of the PS II complex including the oxygen evolving system and Q.) It was reasoned that the oxidation of Q was rate limiting in the conversion of $S'_2 \rightarrow S_3$.

To further explore the effect of bicarbonate depletion on electron transport, diuron induced chlorophyll a fluorescence change was measured as a function of pre-flash number[4]. In this system, bicarbonate sufficient chloroplasts show an oscillating fluorescence pattern with high yields on odd flashes and low yields on even flashes. Bicarbonate depleted chloroplasts had high fluorescence yields without an oscillating pattern. This was interpreted as a block between the R and PQ step. In further experiments without diuron addition, it was shown that the Q^-R^{2-} state could be accumulated by giving three short light flashes to bicarbonate-depleted thylakoids. Measuring chlorophyll a fluorescence, the half-time of the Q^-R^{2-} decay rate was found to be 100 to 200 ms in bicarbonate depleted samples, while it was approximately

1 ms in bicarbonate sufficient chloroplasts, thus indicating a major bottleneck in electron transport alleviated by bicarbonate addition. Siggel *et al.*[19] measured the decay of the reduced quinone pool (R^{2-} and PQ^{2-}) using absorption measurements and found that control chloroplasts had a half-time of decay of 26 ms, while bicarbonate-depleted chloroplasts had a half-time of 100 ms. Thus, in bicarbonate depleted chloroplasts the rate limiting step in electron transport was large enough to account for the overall inhibition of Hill reaction activity.

Evidence cited above has shown that bicarbonate is a reversible activator of the PS II reaction center and of two points within electron transport on the reducing side of PS II. Bicarbonate activation appears to be an essential requirement for the Hill reaction, and specificity for bicarbonate appears to be absolute. Good[3] implied that the active species was the bicarbonate ion because of the synergism of chloride and formate ions with the bicarbonate ions is producing a greater enhancement of Hill reaction activity. More recent evidence[10] has shown that formate acts as a competitive inhibitor with respect to bicarbonate in the activation of the Hill reaction, supporting the contention that the ionic species may be the activator. It would be of interest to know whether formate is binding to the same site. Other carboxylic acids with different pka's may be useful in determining whether the activator site binds ions. Kinetic experiments determining the rate of activation by bicarbonate as compared to carbon dioxide may be difficult since: (I) the pH optimum for the Hill reaction is close to the pKa of carbonic acid ; (II) the rate of binding may be slow so the ionic and gaseous species equilibrate before full activation is achieved ; and (III) the activating site may be buried within the membrane allowing only gaseous carbon dioxide to penetrate. In this case, the activation kinetics would measure the rate of membrane penetration and not the reactivity to the activating site. Sarojini and Govindjee[18] have recently observed that when CO_2 is injected to bicarbonate depleted chloroplasts kept at 5 °C, there is an almost instantaneous stimulation of the Hill reaction, whereas if bicarbonate ion is injected, there is an approximate lag of eight seconds. These results suggest that CO_2 may be the active species that binds to the membrane and that the binding site may be closer to the outer than the inner side of the membrane. Khanna *et al.*[11] have speculated that the effect of CO_2 on the photochemical properties of PS II occurs via CO_2 binding to a polypeptide of the PS II complex which functions as an electron carrier on the reducing

side of PS II. This suggestion is consistent with their observation on the alteration of the binding affinity of ^{14}C-atrazine to the thylakoids upon CO_2-depletion ; atrazine is a PS II inhibitor and has been suggested to bind to the "R-protein" located on the outer (not the inner) side of thylakoid membrane.

Since the activating site binds bicarbonate tightly, it lends itself to active site directed reagent and bicarbonate protection studies. [^{14}C] labeled cyanate is available commercially and could prove useful in purifying a labeled activating site. Another possible method for labeling the site permanently is to bind [^{14}C] bicarbonate to the high affinity sites and stabalize the attachment with a methylating agent, such as diazomethane. The labeled moieties could then be purified by standard methods.

Whether bicarbonate activation of PS II activity is important *in vivo* is difficult to judge. The tight binding of bicarbonate would seem to assure full activation under most conditions. Yet, the binding affinity is known to be inhibited by high pH and by formate ions. Further evidence with regard to other modulators of bicarbonate binding will shed light on the possible importance of bicarbonate activation of the Hill reaction *in vivo*.

The preparation of this short review was supported by financial assistance from USDA (5901-40) and NSF (24532).

REFERENCES

1. Batra, P.O. and Jagendorf, A. T. (1965) *Plant Physiol.*, **40** : 1074-1079.
2. Cohen, W. S. and Jagendorf, A. T. (1974) *Plant Physiol.*, **53** : 220-223.
3. Good, N. E. (1963) *Plant Physiol.*, **38** : 298-304.
4. Govindjee, Pulles, M. P. J., Govindjee, R., Van Gorkom, H. J. and Duysens, L. N. M. (1976) *Biochim. Biophys. Acta.*, **449** : 602-605.
5. Govindjee and Van Rensen, J. J. S. (1978) *Biochim. Biophys. Acta.*, **505** : 183-213.
6. Izawa, S. (1962) *Plant Cell Physiol.*, **3** : 221-227.
7. Jursinic, P. and Govindjee (1977) *Biochim. Biophys. Acta.*, **461** : 253-267.
8. Jursinic, P., Warden, J. and Govindjee (1976) *Biochim. Biophys. Acta.*, **440** : 322-330.
9. Kelly, P.M. and Izawa, S. (1978) *Biochim. Biophys. Acta.*, **502** : 198-210.
10. Khanna, R., Govindjee and Wydrzynski, T. (1977) *Biochim. Biophys. Acta.*, **462** : 208-214.

11. Khanna, R., Pfister, K., Van Rensen, J. J. S., Keresztes, A. and Govindjee (1980) *Biochim. Biophys. Acta.*, (*in press*).
12. Laing, W. A., Ogren, W. L. and Hageman, R. H. (1974) *Plant Physiol.*, **54** : 678-685.
13. Lorimer, G. H., Badger, M. R. and Andrews, T. J. (1976) *Biochim.*, **15** : 529-536.
14. Nelson, N., Nelson, H. and Racker, E. (1972) *J. Biol. Chem.*, **247** : 6506-6510.
15. Pon, N. G., Rabin, B. R. and Calvin, M. (1963) *Biochem. Z.*, **388** : 7-19.
16. Punnett, T. (1965) *Plant Physiol.*, **40** : 1283-1284.
17. Punnett, T. and Iyer, R. V. (1964) *J. Biol. Chem.*, **239** : 2335-2339.
18. Sarojini, G. and Govindjee (1980) *Proc. 5th International Congress on Photosynthesis, Greece ; Biochim. Biophys Acta.*, (*in press*).
19. Siggel, U., Khanna, R., Renger, G. and Govindjee (1977) *Biochim. Biophys. Acta.*, **462** : 196-207.
20. Stemler, A. (1977) *Biochim. Biophys. Acta.*, **460** : 511-522.
21. Stemler, A. and Govindjee (1973) *Plant Physiol.*, **52** : 119-123.
22. Stemler, A. and Govindjee (1974) *Plant Cell Physiol.*, **15** : 533-544.
23. Stemler, A., Babcock, G. T. and Govindjee (1974) *Proc. Natl. Acad. Sci.*, **71** : 4679-4683.
24. Stern, B. K., and Vennesland, B. (1960) *J. Biol. Chem.*, **235** : PC 51-53.
25. Stern, B. K. and Vennesland, B. (1962) *J. Biol. Chem.*, **237** : 596-602.
26. Warburg, O. and Krippahl, G. (1958) *Z. Naturforsch.*, **13b** : 509-514.
27. Warburg, O. and Krippahl, G. (1960) *Z. Naturforsch.*, **15b** : 367-369.
28. Warburg, O., Krippahl, G., Gewitz, H. S. and Volker, W. (1959) *Z. Naturforsch.*, **14b** : 712-724.
29. West, J. and Hill, R. (1967) *Plant Physiol.*, **42** : 819-826.
30. Wydrzynski, T. and Govindjee (1975) *Biochim. Biophys. Acta.*, **387** : 403-408.

Note added in proof :

At the proof stage, the present authors became aware of additional new research in the area of this review ; these include : (a) Stemler, A. (1980) *Plant Physiol.*, **65** : 1160-1165 ; (b) Vermaas, W. F. J. and Van Rensen, J. J. S. (1980), Proc. 5th International Congress on Photosynthesis, Greece ; (c) Stemler, A. (1980), Proc. 5th International Congress on Photosynthesis Research ; and (d) Van Rensen, J. J. S. and Kramer, H. J. M. (1979) *Plant Sci. Lett.*, **17** : 21-27. The readers should consult these publications for newer progress in this field.