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SITE OF BICARBONATE EFFECT IN HILL REACTION

EVIDENCE FROM THE USE OF ARTIFICIAL ELECTRON ACCEPTORS AND DONORS

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SUMMARY

Using artificial electron donors and acceptors, it is shown here that the major HCO_3^- effect in the Hill reaction is after the "primary" electron acceptor (Q) of Photosystem II and before the site of action of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (at the plastoquinone pool). Chloroplasts in the presence of both 3-(3',4'-dichlorophenyl)-1,1-dimethylurea, which blocks electron flow from the reduced primary acceptor Q^- to the plastoquinone pool, and silicomolybdate, which accepts electrons from Q^- , show no significant bicarbonate stimulation of electron flow. However, a 6–7-fold stimulation is clearly observed when oxidized diaminodurene, as an electron acceptor, and dibromothymoquinone, as an inhibitor of electron flow beyond the plastoquinone pool, are used. In the same chloroplast preparation no measurable effect of bicarbonate is observed in a Photosystem I reaction as monitored by electron flow from reduced diaminodurene to methyl viologen in the presence of 3-(3',4'-dichlorophenyl)-1,1-dimethylurea. The insensitivity of the bicarbonate effect to uncouplers of photophosphorylation and the dependence of this effect on the presence of a weak acid anion and on external pH are also reported.

INTRODUCTION

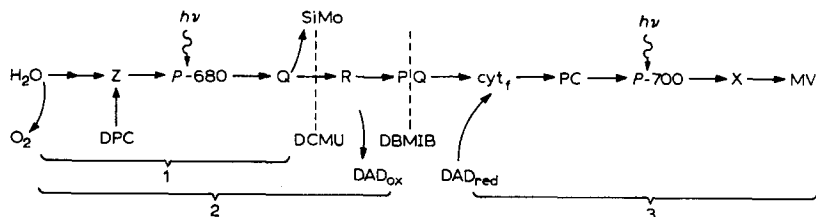
Investigations [1–5] on the role of bicarbonate (HCO_3^-) in the Hill reaction have shown that this anion is required for Photosystem II reactions. When bicarbonate is added to samples previously depleted of bicarbonate a large increase (4–10-fold) in oxygen evolution is observed. Wydrzynski and Govindjee [5] showed that at least one site of bicarbonate action is on the reducing side of Photosystem II. Recent experi-

Abbreviations: DAD_{ox} , oxidized diaminodurene, DAD_{red} , reduced diaminodurene; DBMIB, dibromothymoquinone or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenol indophenol; DPC, diphenylcarbazide; MV, methyl viologen; SiMo, silicomolybdate.

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ments, using chlorophyll *a* fluorescence as a tool for measuring the different steps on the reducing side of Photosystem II, demonstrated that the site of inhibition of Hill reaction by bicarbonate depletion is between the "primary" electron acceptor (Q) of Photosystem II and the secondary electron acceptor (R) [6] and more significantly between R and the plastoquinone (PQ) pool [7]. In this paper we present independent biochemical data showing that the major site of the bicarbonate effect is indeed between Q and the PQ pool.

Using appropriate artificial electron donor-acceptor combinations in conjunction with specific inhibitors of electron carriers, the electron transport chain may be isolated into several segments:



where, Z is the first electron donor to the reaction center chlorophyll *P*-680, DPC is an artificial electron donor to Photosystem II, SiMo accepts electrons from Q⁻ [8, 9], R is a two-electron accumulating intermediate [10], DCMU is an inhibitor that blocks electron flow from Q⁻ to R, DAD_{ox} accepts electrons somewhere before the plastoquinone pool [11], DBMIB is an inhibitor of electron flow at PQ [12], cyt_f is cytochrome *f*, PC is plastocyanin, DAD_{red} acts as an artificial electron donor to Photosystem I [13], *P*-700 is reaction center chlorophyll of Photosystem I, X is the primary electron acceptor of Photosystem I, and MV accepts electrons from X⁻. Using various partial reactions, indicated by 1, 2 and 3 in the above scheme, we determined the bicarbonate effect on these segments of the electron transport chain.

In order to further characterize the bicarbonate effect on the Hill reaction, we measured the sensitivity of the bicarbonate effect to uncouplers of photophosphorylation, to pH and to the presence of a competing weak acid anion (formate). We find that the HCO₃⁻ effect has a pH optimum around 6.5–6.8. We also demonstrate that the stimulation of the Hill reaction is not caused by an enhancement of photophosphorylation.

MATERIALS AND METHODS

Chloroplasts were isolated from leaves of fresh spinach (*Spinacea oleracea*) or Romain lettuce (*Lactuca sativa*) and depleted of bicarbonate by the method of Stemler and Govindjee [1] as modified by Wydrzynski and Govindjee [5].

Electron transport was measured as oxygen evolution using [Fe(CN)₆]³⁻, SiMo or DAD_{ox} as electron acceptors or as oxygen uptake using methyl viologen as an electron acceptor, with a Clark (platinum/Ag-AgCl₂) electrode and a Yellow Springs Oxygen monitor (Model 53). The signal was recorded on an Esterline Angus (Model E11015) recorder. Saturating light (250 mW/cm²) from a tungsten lamp was focused on the chloroplast suspension through a Corning glass CS 3-70 filter. Chlorophyll concentration was determined by the method of Mackinney [14].

For electron flow from H_2O to Q (Reaction 1) 25 μM SiMo and 5 μM DCMU were used. This electron transport stops within 1 min after the addition of SiMo; therefore we monitored only the initial rates after additions (see ref. 8 for details). For electron flow from H_2O to PQ (Reaction 2), 0.5 mM DAD and 0.5 μM DBMIB were used; DAD was kept oxidized with 0.5 mM $[Fe(CN)_6]^{3-}$ (see ref. 11 for details). For electron flow involving only system I (Reaction 3), reduced DAD (0.5 mM DAD plus 2.0 mM ascorbate) was the electron donor, 50 μM methyl viologen the electron acceptor and 1 μM DCMU the electron flow inhibitor after Q (see refs. 13 and 15 for details).

For conducting experiments in the presence of reduced amounts of formate, the chloroplast samples containing formate (added during the HCO_3^- depletion procedure) were washed once and resuspended in a formate-free medium.

RESULTS AND DISCUSSION

Bicarbonate stimulation in the presence of uncouplers of photophosphorylation. Bicarbonate causes an enhancement of photophosphorylation [16, 17] and changes the conformation of the coupling factor [18]. It is necessary to differentiate between the direct stimulation of electron transport by bicarbonate and any indirect stimulation that may be produced by an enhancement of photophosphorylation by bicarbonate. We measured the bicarbonate effect in the presence of several uncouplers of photophosphorylation (Table I). This effect ranges from 6- to 10-fold, but no change in the effect on oxygen evolution is observed when 1 mM NH_4Cl (Table I (1)), 0.3 mM methylamine \cdot HCl (Table I (2)), 5 μM nigericin (Table I (3)) or 1 μM gramicidin D (Table I (4)) is included in the reaction mixture. Obviously our samples are already uncoupled due to the high concentration of salts used in the depletion medium. Thus,

TABLE I

BICARBONATE EFFECT IN THE PRESENCE OF UNCOUPLERS OF PHOTOPHOSPHORYLATION: RATES OF O_2 EVOLUTION

The 2 ml reaction mixture consisted of 50 mM phosphate buffer (pH 6.8), 100 mM sodium formate, 100 mM NaCl and 0.5 mM ferricyanide. Concentrations of uncouplers used were: NH_4Cl (1 mM), methylamine \cdot HCl (0.3 mM), nigericin (5 μM) and gramicidin D (1 μM). Spinach chloroplasts containing 33 μg chlorophyll/ml suspension were used. Average of three experiments is shown.

	Electron transport ($\mu equiv./mg$ chlorophyll per h)		Ratio
	$-HCO_3^-$	+10 mM HCO_3^-	+ HCO_3^- / $-HCO_3^-$
(1) Control	26 \pm 3	171 \pm 9	6.6
Plus NH_4Cl	24 \pm 4	163 \pm 7	6.8
(2) Control	22 \pm 3	134 \pm 14	6.1
Plus methylamine \cdot HCl	23 \pm 3	147 \pm 18	6.4
(3) Control	22 \pm 4	165 \pm 15	7.5
Plus nigericin	21 \pm 1	144 \pm 5	6.9
(4) Control	13 \pm 1	135 \pm 4	10.4
Plus gramicidin D	12 \pm 1	125 \pm 7	10.4

the bicarbonate effect we have studied is not related to its effects on photophosphorylation.

Influence of external pH on bicarbonate stimulation. The effect of suboptimal concentration (2.0 mM) of bicarbonate as a function of pH (5.0–9.0) shows that a much larger stimulation in Hill activity is observed around pH 6–7 (Fig. 1); a 2-fold effect is seen at pH 6.8 but none at pH 5.0. Our studies confirm the measurements of Stemler and Govindjee [1] at pH 5.8 and 6.8. A plot of relative concentrations of CO_2 , HCO_3^- and CO_3^{2-} [19] as a function of pH shows that the bicarbonate species predominates in the pH 6–7 range where we have the maximal effect. It is likely that the HCO_3^- is the active species in stimulating Hill reaction. However, the affinity of HCO_3^- to the membrane component(s) may be different at different pH values. Thus, no definite conclusion can be made regarding the active species involved.

In going from pH 6.4 to 6.8 (Fig. 1), a decline in O_2 evolution is observed both in the absence and the presence of bicarbonate in our samples. These data do not agree with the earlier data of West and Hill [20] (also see Good et al. [21]); we do not know the reason for this discrepancy.

Bicarbonate concentrations required for the stimulation of Hill reaction. The requirement of high salt concentrations for the maximum bicarbonate effect is not fully explained [20, 22]. If we assume that HCO_3^- is the active moiety, other anions such as formate may compete with it for binding sites in the thylakoid membrane [1]. Since the need for HCO_3^- is specific [22], such competitive action by other anions may effectively reduce the stimulatory effect of HCO_3^- . To check this we measured the HCO_3^- stimulation of Hill reaction in the absence of high concentration of formate.

The Hill activity (O_2 evolution) measured as a function of bicarbonate concentration is shown in Fig. 2. In the presence of 100 mM formate in the assay medium

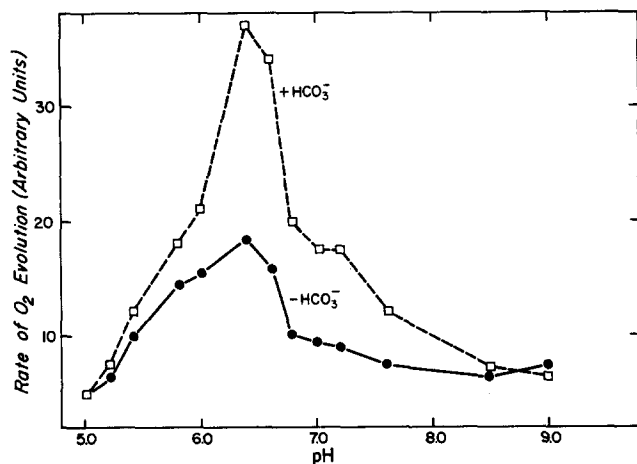


Fig. 1. Rate of oxygen evolution as a function of pH in the presence (\square) and absence (\bullet) of HCO_3^- . The reaction mixture (2 ml) contained 50 mM phosphate buffer, 100 mM sodium formate, 100 mM NaCl and 0.5 mM $[\text{Fe}(\text{CN})_6]^{3-}$. Upper curve (\square - \square), with 2.0 mM NaHCO_3 ; lower curve (\bullet - \bullet), without NaHCO_3 . Chlorophyll concentration, 33 $\mu\text{g}/\text{ml}$ suspension; light intensity, 250 mW/cm^2 ; maximum rate of electron flow, 150 $\mu\text{equiv.}/\text{chlorophyll per h}$.

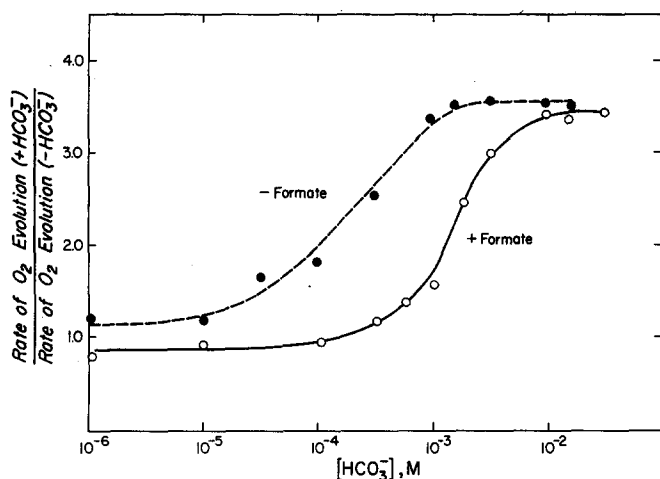


Fig. 2. Effect of bicarbonate on O_2 evolution with and without formate in the assay medium. In the plus-formate samples, the concentration of formate was 100 mM. For minus-formate samples, the formate-containing chloroplasts were washed once and resuspended in a formate-free medium. pH of the medium, 6.8; maximum rate of electron flow, 250 μ equiv./mg chlorophyll per h. Other conditions as described in the legend of Fig. 1.

the amount of bicarbonate required to see half-maximum stimulation is in the range of 10^{-3} M. When formate was omitted from the assay mixture the half-maximum stimulation is in the range of 10^{-4} M. These results confirm the unpublished observations of Stemler and Govindjee (cited in ref. 1) on the stimulation of Hill reaction by bicarbonate in the absence of acetate. Thus, it appears that the HCO_3^- phenomena studied here may have physiological significance. Formate was left in the reaction mixture in all our experiments to reduce the effect of contamination by atmospheric CO_2 .

Site of bicarbonate effect. The bicarbonate effect in various partial reactions (see Introduction) is shown in Table II.

H_2O to silicomolybdate system (Reaction 1). This abbreviated reaction involves the oxygen evolution apparatus, the photochemistry of Photosystem II and the primary electron acceptor (Q). No significant stimulation of O_2 evolution was observed upon the addition of 10 mM HCO_3^- to bicarbonate-depleted samples (Table II (1)). This implies that no major site of HCO_3^- action is on the oxidizing side of Photosystem II. The silicotungstate system [8] showed similar results.

Jursinic et al. [6] and Stemler et al. [4] have shown that, in addition to the effect mentioned above, absence of HCO_3^- causes a reversible inactivation of up to 40% of the Photosystem II reaction center activity. Siggel et al. [23] have found that this inactivation of reaction centers ranges from 5 to 40%. Thus, the absence of the HCO_3^- effect in the $H_2O \rightarrow SiMo$ reaction at saturating light intensities may be due to the low inactivation of reaction center II in our preparations. Alternatively, silicomolybdate somehow overcomes this inactivation.

H_2O to oxidized diaminodurene (Reaction 2). In this reaction, 7–8-fold enhancement of O_2 evolution is observed when 10 mM $NaHCO_3$ is added to samples depleted

TABLE II

EFFECT OF BICARBONATE ON VARIOUS ISOLATED ELECTRON TRANSPORT SYSTEMS

Chloroplasts containing 33 μg chlorophyll/ml were illuminated in a continuously stirred reaction mixture (2 ml) containing 50 mM phosphate buffer (pH 6.8), 100 mM sodium formate, 100 mM NaCl and the indicated donor and acceptor system. These systems were: (1) $\text{H}_2\text{O} \rightarrow \text{SiMo}$; 5 μM DCMU and 25 μM SiMo. (2) $\text{H}_2\text{O} \rightarrow \text{DAD}_{\text{ox}}$; 0.5 mM DAD, 0.5 mM $(\text{Fe}(\text{CN})_6)^{3-}$ and 0.5 μM DBMIB. (3) $\text{DAD}_{\text{red}} \rightarrow \text{MV}$; 50 μM MV, 0.5 mM DAD, 2.0 mM sodium ascorbate and 1 μM DCMU. When SiMo or DAD_{ox} was the electron acceptor, electron transport was observed as O_2 evolution. When MV was the acceptor, electron transport was followed as O_2 uptake. All data have been converted to μ equivalents/mg chlorophyll per h. Average of three experiments is shown.

System (see scheme in Introduction)	Electron transport ($\mu\text{equiv./mg}$ chlorophyll per h)	
	$-\text{HCO}_3^-$	+10 mM HCO_3^-
(1) H_2O to silicomolybdate	117 \pm 16	108 \pm 17
(2) H_2O to oxidized diaminodurene	12 \pm 1	90 \pm 2
(3) Reduced diaminodurene to methyl viologen	662 \pm 12	673 \pm 16

of HCO_3^- , just as in the H_2O to $(\text{Fe}(\text{CN})_6)^{3-}$ system (Table II (2)). DAD_{ox} must intercept electrons before they reach the last molecule in the plastoquinone pool. Since DBMIB does not allow electron flow out of the reduced plastoquinone pool [12], we conclude that HCO_3^- acts somewhere between Q and PQ.

Reduced diaminodurene to methyl viologen system (Reaction 3). No significant effect of HCO_3^- is observed (Table II (3)) in this Photosystem I reaction. This shows that there is no site of inhibition by bicarbonate depletion in the system I reaction except at the phosphorylation level [16, 17].

CONCLUDING REMARKS

We do not yet understand the mechanism of HCO_3^- action. One possible mode of action of HCO_3^- may be through its indirect effect on the conformation of the R complex (R is a quinone, ref. 24). The absence of HCO_3^- probably inactivates the R complex in such a way that the electrons from Q are unable to be transferred to R and from R to the PQ pool. Conformational changes may be brought about by the binding of HCO_3^- to the membrane components to which R is attached.

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REFERENCES

- 1 Stemler, A. and Govindjee (1973) *Plant Physiol.* 52, 119–123
- 2 Stemler, A. and Govindjee (1973) *Photochem. Photobiol.* 19, 227–232
- 3 Stemler, A. and Govindjee (1974) *Plant Cell Physiol.* 15, 533–544

- 4 Stemler, A., Babcock, G. and Govindjee (1974) *Proc. Natl. Acad. Sci. U.S.* 71, 4679–4683
- 5 Wydrzynski, T. and Govindjee (1975) *Biochim. Biophys. Acta* 387, 403–408
- 6 Jursinic, P., Warden, J. and Govindjee (1976) *Biochim. Biophys. Acta* 440, 322–330
- 7 Govindjee, Pulles, M. P. J., Govindjee, R., van Gorkom H. J. and Duysens, L. N. M. (1976) *Biochim. Biophys. Acta* 449, 602–605
- 8 Zilinskas, B. A. and Govindjee (1975) *Biochim. Biophys. Acta* 387, 306–319
- 9 Giaquinta, R. T. and Dilley, R. A. (1975) *Biochim. Biophys. Acta* 387, 288–305
- 10 Velthuys, B. R. and Amesz, J. (1974) *Biochim. Biophys. Acta* 333, 85–94
- 11 Ouitrakul, R. and Izawa, S. (1973) *Biochim. Biophys. Acta* 305, 105–118
- 12 Böhme, H., Reimer, S. and Trebst, A. (1971) *Z. Naturforsch.* 26b, 341–352
- 13 Izawa, S., Gould, J. M., Ort, D. R., Felker, P. and Good, N. E. (1973) *Biochim. Biophys. Acta* 305, 119–128
- 14 Mackinney, G. (1941) *J. Biol. Chem.* 140, 315–322
- 15 Good, N. E. and Hill, R. (1955) *Arch. Biochim.* 57, 355–366
- 16 Punnett, T. and Iyer, R. V. (1964) *J. Biol. Chem.* 239, 2335–2339
- 17 Batra, P. P. and Jagendorf, A. T. (1965) *Plant. Physiol.* 40, 1074–1079
- 18 Nelson, N., Nelson, H. and Racker, E. (1972) *J. Biol. Chem.* 247, 6506–6510
- 19 Fogg, G. E. (1968) *Photosynthesis*, pp. 13–23, American Elsevier Publ. Co., Inc. New York
- 20 West, J. and Hill, R. (1967) *Plant Physiol.* 42, 819–826
- 21 Good, N. E., Izawa, S. and Hind, G. (1966) *Curr. Top. Bioenerg.* 1, 75–112
- 22 Good, N. E. (1963) *Plant Physiol.* 38, 298–304
- 23 Siggel, U., Khanna, R., Renger, G. and Govindjee (1977) *Biochim. Biophys. Acta* 462, 196–207
- 24 Pulles, M. P. J., van Gorkom, H. J. and Willemsen, J. G. (1976) *Biochim. Biophys. Acta* 449, 536–540