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A NEW SITE OF BICARBONATE EFFECT IN PHOTOSYSTEM II OF PHOTOSYNTHESIS: EVIDENCE FROM CHLOROPHYLL FLUORESCENCE TRANSIENTS IN SPINACH CHLOROPLASTS

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Summary

Recent studies on oxygen evolution of corn chloroplast fragments in flashing light [Stemler, A., Babcock, G.T. and Govindjee (1974) Proc. Natl. Acad. Sci. 71, 4679—4683] have shown that the absence of bicarbonate ions increases the turnover time of the Photosystem II reaction center. The rate limiting steps in Photosystem II turnover can be interpreted in terms of reactions either on the oxidizing (electron donor) or reducing (electron acceptor) side of the reaction center. Experiments are reported here that suggest at least one site of bicarbonate action on the reducing side. In Tris-washed spinach chloroplasts (incapable of O_2 evolution), the chlorophyll *a* fluorescence transient in the presence of various artificial electron donors (hydroquinone, diphenylcarbazide, $MnCl_2$ and NH_2OH) and in the absence of bicarbonate ions shows a rapid initial rise; the addition of 10 mM $NaHCO_3$ restores the transient to one characteristic of normal chloroplasts. Furthermore, the transients measured as a function of decreasing bicarbonate concentrations are qualitatively similar to those observed with increasing concentrations of 3-(3, 4-dichlorophenyl)-1, 1-dimethyl urea which imposes a block on the reducing side, rather than to transients observed with increasing concentrations of NH_2OH or prolonged heat treatments, which impose a block on the oxidizing side.

It has recently been shown that the bicarbonate ion is essential in the operation of Photosystem II reactions [1—5]. Large (4—10 fold) increases in oxygen evolution as well as 2, 6-dichlorophenol indophenol or ferri cyanide reduction can be obtained by adding bicarbonate back to previously bicarbonate-depleted chloroplasts. Stemler et al. [4] have established that the absence of bicarbonate increases the Photosystem II turnover time, i.e., the dark time

required, following excitation, before a reaction center may effectively utilize another photon. The rate limiting step in Photosystem II turnover may lie in either the recovery reaction of Q^- to Q or the recovery reaction of Z^+ to Z, where Q and Z refer to the primary electron acceptor and the primary electron donor, respectively [6]. Thus, the effect caused by the absence of the bicarbonate ion can be interpreted either as a block on the reducing (electron acceptor) side or a block on the oxidizing (electron donor) side of Photosystem II.

Based on the absence of a bicarbonate effect on the photoreduction of dichlorophenol indophenol by the artificial electron donor diphenylcarbazide in heat-treated chloroplasts (i.e., chloroplasts unable to evolve oxygen), Stemler and Govindjee suggested that the absence of bicarbonate creates a block on the oxidizing side. The bicarbonate effect on the photoreduction of transient, on the long-term (0.5—5 s) delayed light emission [2] and on the kinetics of oxygen evolution in flashing light [4] were interpreted on this hypothesis. However, the diphenylcarbazide experiment can also be explained by assuming that electron donation by diphenylcarbazide is rate limiting and obscures any bicarbonate effect. In addition we can not exclude the possibility that the reduction of dichlorophenol indophenol by diphenylcarbazide occurs through a mechanism different than the one for the reduction of dichlorophenol indophenol by the natural electron donor.

The fast chlorophyll *a* fluorescence transient ($\leqslant$ 1 s) shows a rapid initial rise in the absence of bicarbonate. Upon addition of saturating amounts of the bicarbonate ion (10 mM), the initial rise in slowed down and the transient shows the usual biphasic kinetics characteristic of dark-adapted chloroplasts. If the absence of bicarbonate creates a block on the oxidizing side, then the restoration of electron flow through the reaction center by an artificial electron donor, such as the hydroquinone-ascorbate couple, should restore the normal transient in bicarbonate-depleted chloroplasts. However, we were unable to affect the fast transient at various hydroquinone-ascorbate concentrations in bicarbonate-depleted chloroplasts (data not shown), suggesting a site of bicarbonate action after the site of electron donation by hydroquinone-ascorbate.

Since added electron donors may not be able to compete well in the presence of the active natural donor system [7] (there is still some oxygen evolution in the absence of bicarbonate) we used chloroplasts which could not evolve oxygen. Spinach chloroplasts isolated in a high salt medium were Tris treated [8] to remove all oxygen-evolving capacity (details are given in the legend to Fig.1). Tris-washed chloroplasts show no variable fluorescence which can be restored by the addition of various electron donors [9]. Optimal concentrations of hydroquinone (250 μM) diphenyl carbazide (2 mM) and hydroxylamine (25 mM) completely restored the variable fluorescence in our system (Fig.1a). We were able to obtain only a partial restoration of the variable fluorescence with 2 mM $MnCl_2$; at high concentrations the manganese precipitated out of solution.

In Tris-washed chloroplasts with the various electron donors the bicarbonate effect is still present on the variable fluorescence (Fig. 1b—c).

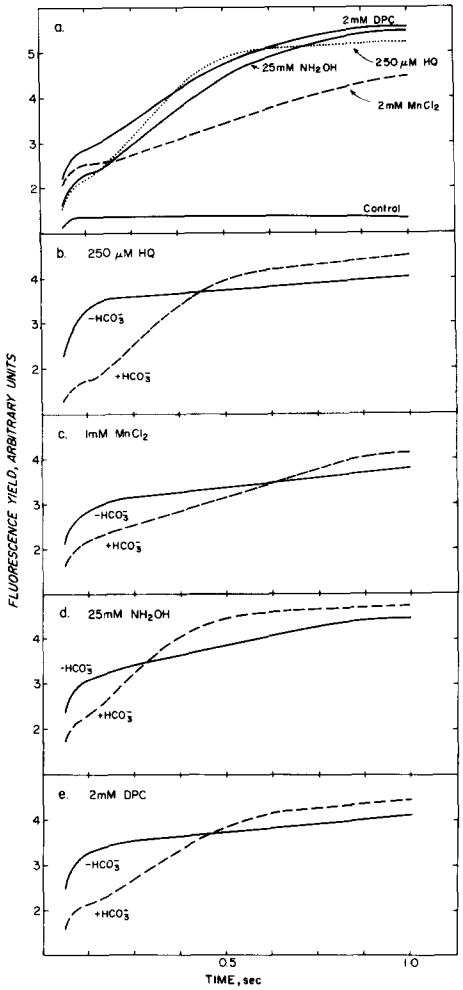

Fig.1. Time course of chlorophyll *a* fluorescence in Tris-washed chloroplasts under various conditions. (a) Restoration of variable fluorescence using different electron donors. (b, c, d, and e) Bicarbonate effect on the initial rise of the variable fluorescence in the different electron donor systems. In $+HCO_3^-$ transients 10 mM $NaHCO_3$ was added to samples previously depleted of HCO_3^-. Spinach chloroplasts were isolated in 50 mM sodium phosphate buffer, pH 8.0, 200mM NaCl and were osmotically shocked. For Tris-treatment chloroplasts were suspended to a final concentration of 50 μg chlorophyll/ml in 0.8 M Tris buffer, pH 8.0 for 30 min at 4°C. Bicarbonate was depleted in 50 mM phosphate buffer, pH 5.4, 100 mM sodium formate and 175 mM NaCl for 10 min in the dark at room temperature under nitrogen. Assay medium was the same as the depletion medium, pH 6.8. Fluorescence was measured at 685 nm (half-band width, 6.6 nm); excitation, broad band blue light (CS 4-96 and CS 3-73), intensity, $1.2 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. Chlorophyll concentration, 12.5 μg/ml suspension. DPC, diphenylcarbazide.

Similar results were obtained for heat treated chloroplasts using 25 mM NH_2OH as the electron donor. These results, therefore, suggest that at least one site of bicarbonate action is after the point where the artificial donors feed electrons into Photosystem II.

Several intermediates have been postulated between water oxidation and the Photosystem II reaction center based on the differential effects of artificial electron donors in variously inhibited systems [10]. However, it is

probable that at least NH_2OH can feed directly to the reaction center complex [11]. If this is the case, then the site of bicarbonate action monitored by the variable fluorescence must be on the reducing side or at the reaction center itself.

Other qualitative data support the above conclusion. The dependency of the fluorescence transient on decreasing bicarbonate concentrations (Fig. 2a) is remarkably similar to the dependence of the transient on 3-(3, 4-dichlorophenyl)-1, 1-dimethyl urea concentration (Fig. 2b) rather than on NH_2OH concentration or heat treatment (Fig. 2c and d). Since it is widely accepted that dichlorophenyl dimethyl urea blocks the reoxidation of Q^- by the A pool while NH_2OH and heat treatment block electron flow from water to the

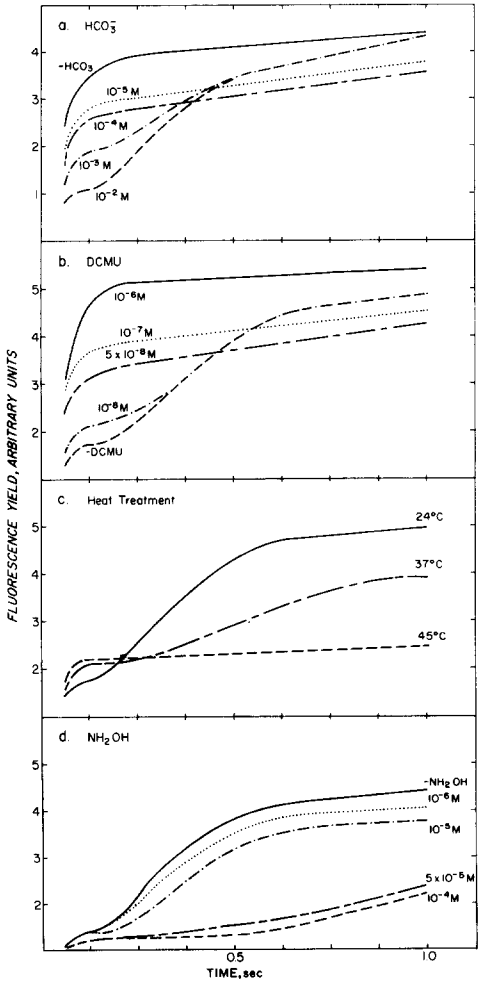

Fig. 2. Comparison of concentration dependence of variable chlorophyll *a* fluorescence on bicarbonate with various System II inhibitory treatments. (a) HCO_3^- depleted chloroplasts at various $NaHCO_3$ concentrations. (b) Normal chloroplasts at various dichlorophenyl dimethil urea (DCMU) concentrations. (c) Normal chloroplasts heat-treated for 1 min (12.5 μg chlorophyll/ml concentration) at different temperatures. (d) Normal chloroplasts at various NH_2OH concentrations. Preincubation time was 5 min in dark. Other details as in Fig. 1.

reaction center, these results are consistent with a site of bicarbonate action on the reducing side of Photosystem II.

Dichlorophenyl dimethyl urea, however, may have a much more complex effect on Photosystem II than simply blocking electron flow between Q and A. Renger [12] has shown that dichlorophenyl dimethyl urea at high concentration acts as an ADRY (Accelerator of Deactivation Reaction of enzyme "Y", labelled "Z" by other investigators) reagent, although bicarbonate does not have a similar function since it was shown by Stemler et al. [4] not to have an accelerating effect on the deactivation of the higher S states involved in O_2 evolution. Bennoun and Li [13] have suggested that dichlorophenyl dimethyl urea, also at high concentrations, could inactivate part of the Photosystem II reaction centers into a non-quenching form, possibly by a direct binding with the reaction center complex. Perhaps bicarbonate has a similar, although reverse, mode of action.

If bicarbonate is acting only on the reducing side, then it is difficult to reconcile the data on the diphenylcarbazide → dichlorophenol indophenol reaction. Table I shows that in our system we get approx. 10-fold stimulation in the rate of dichlorophenol indophenol reduction with the natural electron donor but only a 2-fold increase in Tris-washed chloroplasts with diphenylcarbazide as the electron donor. At a sufficiently high diphenylcarbazide concentration (100 μM) the rate of reaction is comparable with that in the control, so that the diphenylcarbazide → dichlorophenol indophenol reaction itself does not appear to be rate limiting. Thus, the bicarbonate effect is considerably reduced using diphenylcarbazide, consistent with the earlier findings of Stemler and Govindjee. It is interesting to point out, however, that 20 times more diphenylcarbazide is required to restore the variable fluorescence in a sample having the same chlorophyll concentration. The different concentration dependence between restoration of the variable fluorescence and dichlorophenol inophenol photoreduction by diphenylcarbzide in Tris-washed chloroplasts may indicate the two phenoma are non-complementary under some conditions. Harnischfeger [7] has suggested that diphenylcarbazide increases the efficiency of Photosystem II in addition to it function as an electron donor. Obviously, bicarbonate also increases the efficiency of Photosystem II so that we might not expect to see a significant bicarbonate effect in the presence of diphenylcarbazide. To reconcile the

TABLE I

BICARBONATE EFFECT ON DICHLOROPHENOL INOPHENOL REDUCTION IN TRIS-WASHED SPINACH CHLOROPLASTS

Condition	μmol dichlorophenol indophenol/mg Chl/h		$+HCO_3^-/-HCO_3^-$
	$-HCO_3^-$	$+HCO_3^-$	
Control	6.4	62.0	9.7
Tris-washed	0	0	-
+ 10 μM diphenylcarbazide	5.9	12.2	2.07
+ 50 μM diphenylcarbazide	12.9	33.6	2.60
+ 100 μM diphenylcarbazide	27.6	61.2	2.21

Details of chloroplast isolation, Tris-treatment and bicarbonate depletion are given in Fig. 1. Assay medium was same as depletion medium, pH 6.8, plus 50 μM dichlorophenol indophenol. Chlorophyll concentration, 12.5 μg/ml. Photoinduced rates of dichlorophenol indophenol reduction were corrected for dark reductions of dichlorophenol indophenol by diphenylcarbazide. Dark rates were 10—20% of photoinduced rates. Light intensity, saturating.

data on diphenylcarbazide → dichlorophenol indophenol electron flow and the fluorescence data presented here, we will have to suggest that either (1) the mechanism of the above electron flow is different than in other systems and needs a separate interpretation, or (2) bicarbonate affects both the oxidizing and reducing sides of Photosystem II.

It is apparent from this fluorescence data and from the earlier kinetic data of O_2 evolution in flashing light that the bicarbonate affects the primary reactions in photosynthesis. The bicarbonate effect is specific for Photosystem II and does not appear to arise from a general membrane phenomenon. This last point is supported by several observations: (1) The absence of a bicarbonate effect in glutaraldehyde-fixed chloroplasts (2) the absence of an affect on the absorption spectra at room and low (77° K) temperature emissoin spectra (T.W. and G., unpublished) and (3) the lack of any consistent effect on photo-induced 90° light scattering or 540 nm transmission changes (T.W. and G., unpublished). From the results reported here the bicarbonate ion seems to be acting on the reducing side of Photosystem II. It may, however, be acting at the reaction center complex itself, in which case it may become a useful probe in studying the primary reactions of Photosystem II.

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