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Further Studies on the Bicarbonate Stimulation of Photophosphorylation in Isolated Chloroplasts[1]

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ABSTRACT

The bicarbonate effect in stimulating the rate of photophosphorylation by isolated spinach (*Spinacia oleracea* var. Virginia blight-resistant savoy) chloroplasts at a pH below the optimum has been re-examined. Its seasonal nature may be related to the hormonal status of the plants. Bicarbonate anions stimulate adenosine 5′-triphosphate synthesis if added in the final, adenosine 5′-triphosphate-forming stage of either a postillumination or an acid-base experiment. They also stimulate the membrane-bound, Mg^{2+}-dependent adenosine 5′-triphosphatase of chloroplasts, and the Ca^{2+}-dependent adenosine 5′-triphosphatase of detached coupling factor. These and other data point to the interaction between energized thylakoid membranes and the coupling factor as the probable site of action of bicarbonate anions when they stimulate photophosphorylation.

The possible regulation of photophosphorylation by normal leaf components is an area that has received little attention to date. It has been reported that bicarbonate will stimulate photophosphorylation (pH 6.5–7.5) in isolated chloroplasts at specific times of the year (3, 15, 16). Recently, it has been reported that bicarbonate will stimulate the photophosphorylation of chromatophores from *Chromatium* strain D (12). In the present study we have re-examined the seasonal effect of bicarbonate on some of the partial reactions of the phosphorylation mechanism in chloroplasts, with the aim of gaining further insights into the site of the stimulation.

MATERIALS AND METHODS

Preparation of Chloroplasts. Chloroplasts were prepared from greenhouse-grown spinach (*Spinacia oleracea* var. Virginia-blight resistant savoy). Fully expanded leaves from 6- to 8-week-old plants were blended in a medium consisting of 0.8 M sucrose, 20 mM Tricine-NaOH, pH 7.3 or 7.8, and 10 mM NaCl. The chloroplast pellet was washed once by centrifugation (10,000*g* for 10 min) with 10 mM NaCl and finally resuspended at 1 mg/ml Chl in the NaCl solution. Chl was estimated by the method of Arnon (1).

Phosphorylation Assays. Cyclic photophosphorylation with pyocyanine as cofactor was performed as described by Cohen and Jagendorf (5). ATP formation was monitored using radioactive phosphate (2) or following the disappearance of Pi (21). Postillumination ATP synthesis was carried out as described by Jagendorf and Hind (8). Acid-base transition ATP formation was assayed by the method of Jagendorf and Uribe (9).

Activation and Assay of Ca^{2+}- and Mg^{2+}-ATPase Activity. Ca^{2+}-ATPase activity was assayed using the method of McCarty as described by Ryrie and Jagendorf (17); either trypsin or DTT[3] served as the activating agent. Mg^{2+}-ATPase activity was activated and assayed as described by McCarty and Racker (11).

Biochemicals. Bicarbonate solutions were prepared fresh daily; they were titrated to the pH of the reaction mixture with HCl and kept in stoppered flasks prior to use. DTT, kinetin, and trypsin were purchased from Calbiochem. Dio-9 was generously supplied by Dr. R. E. McCarty.

RESULTS

As previously described by Punnett (15) and Batra and Jagendorf (3), addition of bicarbonate to a reaction mixture just prior to illumination can stimulate the rate of pyocyanine-catalyzed photophosphorylation as much as 400% (Table I). The data for Table I were collected in the winter of 1970; the following winter (1971) the stimulation rarely exceeded 60 to 80% of the control values. As reported earlier, by the above workers, the bicarbonate stimulation in the summer and early fall was only on the order of 10 to 25%.

Batra and Jagendorf (3) also observed that addition of bicarbonate tended to inhibit ATP synthesis occurring after illumination in a two-stage, light to dark experiment (8). In their work, the bicarbonate was added only prior to illumination. This inhibition is demonstrated again in the experiment shown in Table II; but if the bicarbonate is added only in the postillumination darkness, together with ADP and phosphate, it stimulates ATP synthesis up to 50% at 8 mM. Similarly, when added with ADP and Pi in the base stage of an acid-base experiment (9) bicarbonate stimulates ATP formation (Table III).

In both instances, the increase in ATP formation occurred when bicarbonate was present in the stage where the conserved energy (presumably a proton gradient) was translated into ATP by the coupling mechanism. We decided, therefore, to investigate the effects of bicarbonate on the coupling factor (CF_1). When the coupling protein is membrane-bound, it ex-

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[3] Abbreviations: DTT: dithiothreitol; X_E: the amount of "energetic intermediate" stored by chloroplasts in the light, as shown by the amount of ATP made in postillumination dark.

hibits a light-induced ATPase activity (11, 14). This hydrolyzing activity can be activated in the presence of an electron transport cofactor (*e.g.* pyocyanine), a thiol-reagent (DTT) and Mg^{2+} ions. Incorporation of bicarbonate in a reaction mixture where activated chloroplasts are hydrolyzing ATP, led to an increase in the rate of hydrolysis compared to controls without bicarbonate (Fig. 1). Addition of bicarbonate to the light-activation stage had little or no effect. The stimulation of Mg^{2+}-ATPase activity appeared to correlate reasonably well with the seasonal stimulation of cyclic photophosphorylation (Table IV).

Bicarbonate also stimulated the Ca^{2+}-ATPase activity of the isolated coupling factor (Table V). The above stimulation may not be related to the stimulation of photophosphorylation, since it was observed at all times of the year.

Bicarbonate stimulated phosphorylation only at ADP concentrations above 0.1 mM, and at phosphate concentrations above 0.75 mM (Fig. 2). Lineweaver-Burk analysis revealed that bicarbonate raised the *Km* values for ADP and phosphate, to a small extent in both cases.

Energy-transfer inhibitors are believed to act close to the site of ATP formation in the energy conservation sequence (6). Addition of the energy-transfer inhibitor Dio-9 (at con-

Table I. *Effect of Bicarbonate on Pyocyanine-catalyzed Photophosphorylation*

The reaction mixture for phosphorylation contained (in 2 ml): 25 mM NaCl, 25 mM MES-NaOH, pH 7.0, 5 mM $MgCl_2$, 2 mM ADP, 2 mM KH_2PO_4, 0.05 mM pyocyanine, and chloroplasts equivalent to 50 μg of Chl. The time of illumination was 3 min, and the temp was 25 C.

$KHCO_3$	ATP Synthesis	Control
mM	*μmoles/mg Chl·hr*	%
0	16.3	100
5	32.7	200
10	65.4	400

Table II. *Effects of Bicarbonate on Postillumination ATP Synthesis*

Postillumination ATP synthesis was measured according to Jagendorf and Hind (8), the light reaction contained in 1.0 ml: 10 mM MES-NaOH, pH 6.3, 50 mM NaCl, 0.05 mM pyocyanine, and chloroplasts equivalent to 250 μg of Chl. The mixture was illuminated with heat-filtered white light (1.5×10^6 ergs/cm²·sec), and the temp was 2 C. After 60 sec, the mixture was injected into a solution (1.0 ml) in the dark containing 100 mM MES-NaOH, pH 7.2, 5 mM $MgCl_2$, 4 mM KH_2PO_4 containing 5×10^5 cpm of ^{32}P and 3 mM ADP. After 30 sec, the reaction was terminated by the addition of trichloroacetic acid.

	$KHCO_3$	ATP Synthesis
	mM	*nmoles/mg Chl*
Light stage	0	14.8
	8	10.1
Dark stage	0	14.8
	2	17.2
	4	19.9
	8	20.3

Table III. *Effect of Bicarbonate on Acid-base Transition ATP Synthesis*

Acid-base induced phosphorylation was assayed as follows. The acid stage (in 1.0 ml) contained 10 mM succinate, pH 3.8, 0.03 mM DCMU, and chloroplasts equivalent to 250 μg of Chl. After 30 sec in the acid stage, the chloroplasts were injected into a base stage (1.0 ml) containing 100 mM MES-NaOH, pH 7.2, 5 mM $MgCl_2$, 2 mM KH_2PO_4 containing 5×10^5 cpm of ^{32}P, 0.2 mM ADP, and enough NaOH to neutralize the acid from the acid stage. After 15 sec the reaction was stopped by the addition of trichloroacetic acid. Bicarbonate was always added to the base stage.

$KHCO_3$	ATP Synthesis
mM	*nmoles/mg Chl*
0	37.1
4	53.9
8	54.8
16	59.9

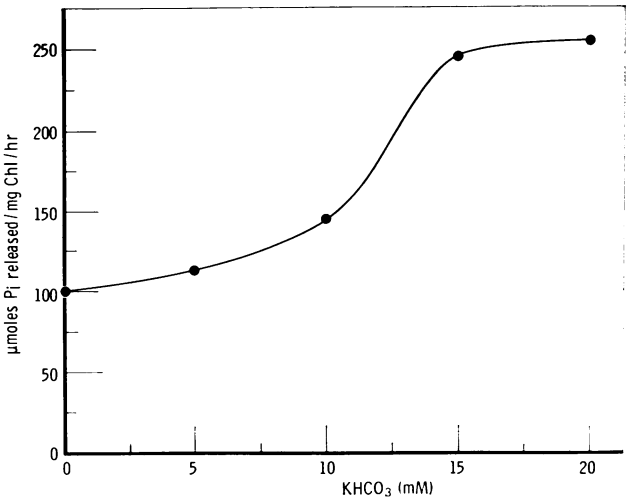

FIG. 1. Effect of bicarbonate on light- and dithiothreitol-activated Mg^{2+}-ATPase activity. The reaction mixture (in 1.0 ml) contained 5 mM ATP, 5 mM $MgCl_2$, 50 mM MES-NaOH, pH 7.0, and light- and DTT-activated, according to McCarty and Racker (11), chloroplasts equivalent to 5 μg of Chl. The temp of incubation was 37 C, and the time of incubation was 20 min.

Table IV. *Seasonal Stimulation of Photophosphorylation and Mg^{2+}-ATPase Activity by Bicarbonate*

Photophosphorylation and Mg^{2+}-ATPase activity were assayed as described in the legends to Table I and to Fig. 1. Bicarbonate was present at 10 mM.

	− $KHCO_3$	+ $KHCO_3$	HCO_3^- Stimulation
			%
Experiment I (June 1970)			
Photophosphorylation[1]	62	100	61
Mg^{2+}-ATPase activity[2]	465	660	42
Experiment II (August 1970)			
Photophosphorylation	122	142	16
Mg^{2+}-ATPase activity	470	526	12
Experiment III (April 1971)			
Photophosphorylation	49	70	43
Mg^{2+}-ATPase activity	99	145	47

[1] μmoles ATP formed/mg Chl·hr.
[2] μmoles ATP hydrolyzed/mg Chl·hr.

Table V. *Effect of Bicarbonate on Trypsin-activated, Ca^{2+}-dependent ATPase Activity*

The reaction mixture (in 1.0 ml) contained 5 mM ATP, 5 mM $CaCl_2$, 25 mM MES-NaOH, pH 7.0 and trypsin-activated chloroplasts equivalent to 4 μg of Chl. The temp of incubation was 37 C, and the reaction was allowed to proceed 20 min before the addition of trichloroacetic acid to a final concentration of 2%.

$KHCO_3$	ATPase Activity	Control
mM	*μmoles P_i released/mg Chl·hr*	%
0	144	100
4	192	133
8	204	142
12	231	160
16	240	166

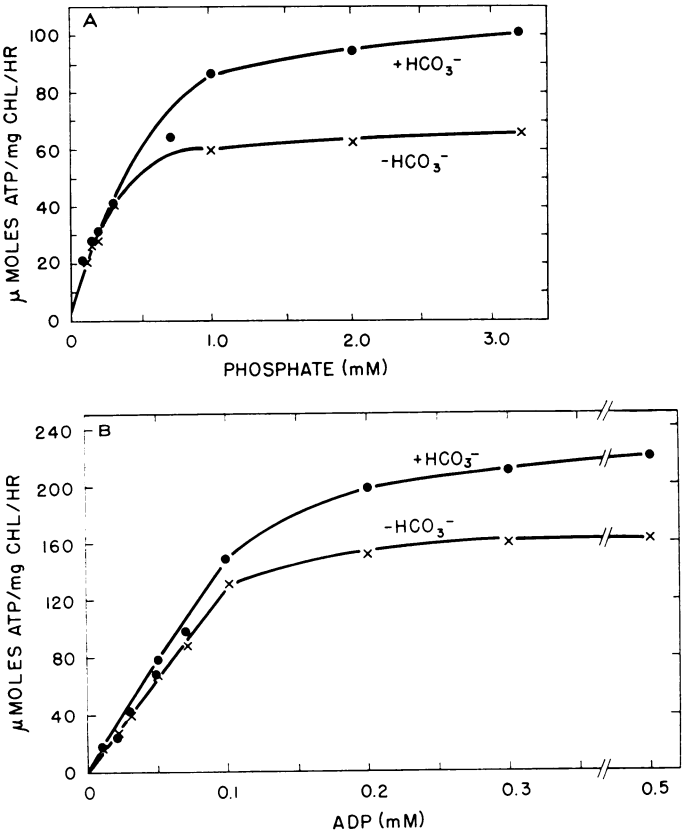

FIG. 2. Kinetic analysis of the effects of bicarbonate stimulation on photophosphorylation. Phosphorylation was measured in a reaction mixture which contained in a volume of 2.0 ml, 25 mM MES-NaOH, pH 7.0, 25 mM NaCl, 5 mM $MgCl_2$, and 0.03 mM pyocyanine together with the following components: A: 2 mM ADP, variable Pi, and 60 μg of Chl; B: variable ADP, 2 mM Pi, and 60 μg of Chl. Each reaction mixture contained 5×10^5 cpm of ^{32}P. The time of illumination in A was 90 sec; in B, 45 sec. When added, bicarbonate was present at 16 mM.

centrations that inhibited phosphorylation 50%) had little or no effect on the stimulation of photophosphorylation by bicarbonate (Table VI).

Various attempts were made to increase the extent of the bicarbonate stimulation at times of the year when the effect was small. Spinach, pea, and oat plants were grown in environmental chambers with short days and cool nights. Spinach and oat plants were also grown under yellow filters as described by Punnett (15). The above attempts were all unsuccessful.

We also examined subchloroplast particles (prepared by sonication) that were made in February and stored at −100 C until July. The rates of ATP synthesis in the stored particles were comparable to those of fresh chloroplasts, but bicarbonate stimulation was only about 30%.

We also tested the effects of plant hormones for possible interaction with the bicarbonate effect. Chloroplasts were prepared, resuspended, and assayed in the presence of 10 μg/l of kinetin, or, alternatively, the kinetin was added only to the assay medium for phosphorylation. Table VII shows that addition of kinetin to a reaction mix for phosphorylation slightly stimulated ATP formation but did not markedly affect the bicarbonate stimulation. The presence of kinetin throughout led to a 34% increase in ATP formation, and bicarbonate then caused only 10% further stimulation. The stimulatory effect of kinetin on cyclic photophosphorylation is similar to the effect of indoleacetic acid, on phosphorylation reported by Tamas *et al.* (20).

DISCUSSION

The observation that bicarbonate stimulates the formation of ATP when present in the dark stage of an X_E experiment (Table II) is in agreement with the effect of bicarbonate on "one-stage" phosphorylation. The inhibitory action of bicarbonate when it is present in the light stage is probably related to a faster efflux of protons in the dark in the presence of bicarbonate (Jagendorf [7] and unpublished data). Since postillumination ATP synthesis depends on the maintenance of a store of internal protons, a substance which causes faster loss of protons should decrease the amount of ATP synthesized.

The stimulatory effects of bicarbonate on ATP synthesis appear to be related to an effect on the coupling mechanism or perhaps more specifically the coupling protein (CF_1). Recently,

Table VI. *Effect of DIO-9 on the Bicarbonate Stimulation of Cyclic Photophosphorylation*

Cyclic photophosphorylation was measured as described in the legend to Table I. Bicarbonate was present at 15 mM.

	ATP Formation		HCO_3^- Stimulation
	− $KHCO_3$	+ $KHCO_3$	
	μmoles ATP/mg Chl·hr		%
Experiment I			
Control	192	316	64
Control + 3 μg/ml Dio-9	100	183	83
Experiment II			
Control	125	200	78
Control + 4 μg/ml Dio-9	55	98	78

Table VII. *Effect of Kinetin on the Bicarbonate Stimulation of Cyclic Photophosphorylation*

Cyclic photophosphorylation was measured as described in the legend to Table I. Kinetin was present at 10 μg/l.

$KHCO_3$	ATP Synthesis		
	− Kinetin	+ Kinetin (reaction mix only)	+ Kinetin (grinding medium and reaction mix)
mM	*μmoles/mg Chl·hr*		
0	35.4	42.5	47.0
15	54.3	58.1	51.9

Nelson *et al.* (13) have shown that bicarbonate stimulates the residual Mg^{2+}-ATPase activity of detached and purified CF_1 3- to 4-fold. They also found similar results with the organic acid maleate, which partially replaces bicarbonate in stimulating ATP formation with pyocyanine as a cofactor (15). Nelson *et al.* (13) have suggested that bicarbonate (and maleate) may increase the Mg^{2+}-ATPase activity of the purified enzyme by changing the conformation of the enzyme, which would result in more efficient catalytic action.

Energy-dependent conformational changes of membrane-bound CF_1 have been demonstrated by Ryrie and Jagendorf (18, 19) by measuring tritiation of the molecule when it is energized. Preliminary experiments, in collaboration with Dr. Ryrie, indicated that the degree of tritium label in CF_1 increased 2-fold when the assay was performed in the presence of bicarbonate.

The mechanism for phosphorylation can be separated into at least three stages: buildup of the high energy state of the chloroplast thylakoid membranes, effect of this state on the coupling factor, and interaction of the coupling factor with ADP and Pi as substrates. While we have no direct information as to the effect of bicarbonate on actual buildup of the high energy state, the experiment in Table II shows that it is inhibitory to those reactions requiring the maintenance of a large protein gradient, as the major component of the high energy state. At the other end, there is little or no indication from kinetic analysis (Fig. 2) of a change in the relationship between the coupling factor and its substrates. Further, Dio-9, which affects CF_1 function directly, does not modify the bicarbonate stimulation (Table VI). These data thus begin to point to the relation between the coupling factor and the energized membrane as a likely site of action of bicarbonate. This concept is supported by the bicarbonate stimulation of tritium incorporation into CF_1, since the measured hydrogen exchange into cryptic groups occurs prior to ATP synthesis and is not inhibited by Dio-9 (19). Increased efficiency at this point would be consistent with the higher P/e_2 ratios for noncyclic phosphorylation reported by Punnett and Iyer (16). It would also be consistent with stimulation of the Mg^{2+}-dependent ATPase (Fig. 1), since both transition of the enzyme to its active form and maintenance in this condition requires the energized condition of the membranes (4, 10).

The kinetin experiments and preliminary observations that chloroplasts from wilted leaves failed to show bicarbonate stimulation indicate that the prior life history of the plants is extremely important in determining the extent of the bicarbonate stimulation of photophosphorylation at pH 7.

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