Bicarbonate Effects on the Hill Reaction and Photophosphorylation[1,2]

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Since the original discovery by Warburg and Krippahl (22, 23), considerable attention has been paid to the CO_2 requirement for the Hill reaction by isolated chloroplasts. Much of the sporadic literature on this subject has been devoted to confirming the existence of the requirement with other kinds of chloroplasts, diverse electron acceptors and somewhat differing conditions (1, 6, 7, 10, 18, 19). Thus, Warburg and Krippahl originally reported a requirement for preillumination during the period of CO_2 depletion, while Stern and Vennesland (18, 19) observed large CO_2 effects without preillumination, and Heise and Gaffron (7) found that preillumination prevented CO_2 depletion from having major inhibitory effects on the quinone reduction by whole cells of *Anacystis*. Both Good (6) and Izawa (10) showed that the absence of CO_2 was more inhibitory at high light intensities than at low intensities, and Abeles et al. (1) observed the CO_2 effect using a mass spectrometer for analysis of O_2 evolution independent of uptake effects.

The most recent investigation, that of Punnett and Iyer (15) extended the observation of CO_2 effects to the phosphorylation accompanying the Hill reaction. Their methods differed strikingly from those of previous workers, however, in that a period of CO_2 depletion of the (oat) chloroplasts was not required in order to see stimulation by added bicarbonate. From their results with phosphorylation as well as the Hill reaction, they postulated that bicarbonate was necessary for the most efficient operation of the photophosphorylation mechanism.

In the present work we have compared the methods of Punnett and Iyer, with those of Warburg and Krippahl as modified by Good (6), using the same chloroplasts and techniques for both types of experiment. Our data indicate that the effect of added bicarbonate is probably different in the 2 systems: the observations of Punnett and Iyer relating to the phosphorylation mechanism, and those of Warburg and Krippahl relating to electron transport in the region of O_2 evolution. We have also demonstrated an inhibitory effect of bicarbonate on the steady state level of a nonphosphorylated, high energy condition of the chloroplasts (8, 16), under the same conditions in which it stimulates photophosphorylation.

Materials and Methods

Chloroplasts were prepared from spinach leaves by grinding and washing in buffer containing 0.4 M sucrose, 0.02 M Tris at pH 8 and 0.01 M NaCl, as described previously (13). The chloroplasts were broken before use, in some experiments, by suspending and washing once in 10 mM NaCl, at 10,000 × *g* for 10 minutes. The pellet was finally suspended in 10 mM NaCl. Chlorophyll was determined by the method of Arnon (2).

Pea chloroplasts were prepared by the method of Good (6), using 0.4 M sucrose, 0.02 M Tris at pH 7.5, 0.01 M NaCl and 0.25 % bovine serum albumen (Janel Fraction V from Gallard Schlesinger Company) as the buffer solution.

Unless otherwise stated, all standard assays for phosphorylation were performed in a 25 ml Erlenmeyer flask maintained at 18 or 20° in a Warburg bath. The reaction volume was either 3 ml containing about 30 to 45 μg of chlorophyll, or 1 ml containing 15 μg of chlorophyll. Illumination was from below from a bank of incandescent lamps beneath the water tank, with a light intensity of 5000 ft-c at flask height. Reaction mixture component concentrations are indicated in the appropriate table and figure legends. $NaHCO_3$ was weighed out, brought to volume and its pH adjusted to either 6.6 or 7.0 immediately before use.

In the CO_2 depletion experiments the reaction mixture containing chloroplasts was placed in a Warburg flask, with P^{32} (where appropriate) and either the ferricyanide or pyocyanine placed in the sidearm. No KOH was used, but the Warburg flasks were flushed with nitrogen gas previously bubbled through a barium hydroxide solution, continuously for 2 hours in room light. At the end of this time the sidearms were opened up, $NaHCO_3$ added, and the sidearms tipped in. In the control flasks nitrogen flushing was continued for this period and during illumination, which was for 4 minutes in all cases. Both in the CO_2 depletion experiments, and with fresh chloroplasts, ferricyanide reduction was estimated by the loss of OD at 420 mμ, in the deproteinized reaction mixtures. Esterified radioactive phosphate was determined variously by

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charcoal adsorption of the ATP^{32} (8), by phase separation of the phosphomolybdate complex as described by Avron (3) or by the precipitation procedure of Sugino and Miyoshi (20).

Estimation of the high energy state X_E was performed as previously described (8) in which chloroplasts, preilluminated at pH 6.5, are injected into ADP, P^{32} and Mg at pH 8 in the dark, and ATP^{32} formation is measured. In the present experiments the phosphate concentration in the dark stage was 2 mM.

Results

As described by Punnett and Iyer (15) for oat chloroplasts, the addition of bicarbonate to a reaction mixture just before illumination can stimulate the rate of a Hill reaction with ferricyanide as electron acceptor. The accompanying phosphorylation is increased to an even greater extent, causing a rise in the P/2e ratio. We have repeated these observations with chloroplasts from both spinach and pea leaves. We should like to note that the effect is most pronounced when the reactions are preformed in a shallow liquid layer in an Erlenmeyer or Warburg flask, rather than in a small and narrow test tube.

Added bicarbonate can affect phosphorylation supported not only by ferricyanide, but also by FMN or pyocyanine as cofactors (table I). Indeed the largest bicarbonate effects are found when pyocyanine is the cofactor. Table I also shows that bicarbonate does not replace the function of an added redox dye, but must act at some other point. Since pyocyanine can support cyclic electron flow, it was important to see whether bicarbonate would stimulate under conditions were O_2 evolution could be rigorously excluded. Table I shows that the bicarbonate effect is not changed by the presence of 10 μM CMU, used under nitrogen, conditions under which the Hill reaction would not be possible (12,21).

Table I. *Effect of Added Bicarbonate on Photophosphorylation Supported by Various Redox Cofactors*

In experiment I the reaction mixture was 1.0 ml, containing in μmoles: Tris-maleate pH 7, 5; NaCl, 17.5; $MgCl_2$, 2.5; phosphate, 1.25; ADP, 1; and either pyocyanine, 0.025, potassium ferricyanide, 0.5 or FMN, 0.05. The reactions were run in air for 3 minutes. Conditions were the same in experiment II except that the reactions were run anaerobically in Warburg flasks, and the phosphate level was raised to 20 μmoles per 1.0 ml. CMU dissolved in methanol was present at 10 μM, and sodium bicarbonate at 8 mM, where indicated. Controls in experiment II contained ethanol in place of the CMU.

Expt	Cofactor	ATP Formation $-HCO_3$	 $+HCO_3$
I	None	1*	3
	Ferricyanide	23	35
	FMN	10	16
	Pyocyanine	41	106
II	Pyocyanine	73	190
	" + CMU	69	198

* μmoles ATP formed per mg chlorophyll per hour.

In other experiments we found that stimulation of phosphorylation was not accomplished by the following organic acids: acetate, phthalate, dimethylglutarate, citrate, benzoate or glycine. The extent of the stimulation by bicarbonate was found to vary with the season of the year, again in agreement with the observations of Punnett and Iyer (15). With spinach chloroplasts in the winter months, bicarbonate stimulations of pyocyanine-supported phosphorylation of up to 160 % were observed; in the late spring some preparations of chloroplasts showed stimulations of only 15 %. In the large majority of experiments the stimulated rates ranged between 1.5 and 2.5 times that of the controls.

Punnett and Iyer (15) used high light intensities in their studies. Analysis of the light intensity curves (figure 1) shows practically identical kinetics for the pyocyanine-supported phosphorylation whether in the presence or absence of added bicarbonate. This localizes the bicarbonate effect on phosphorylation to a limiting dark step. A further consequence of this fact is that bicarbonate will not stimulate if the chlorophyll concentration in the reaction is too high, since this will lower the effective light intensity. Under our present conditions the maxi-

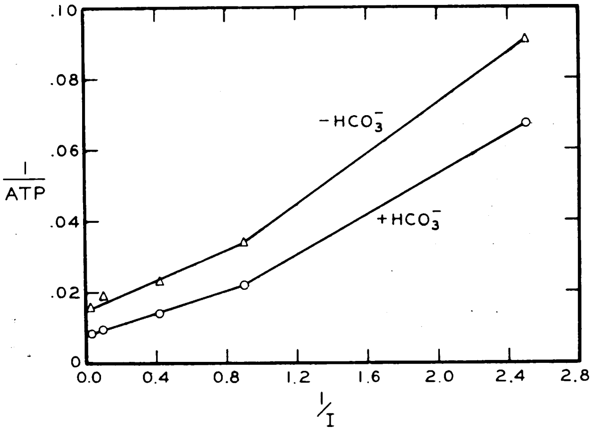

Fig. 1. Relation of bicarbonate effect on photophosphorylation to light intensity. Reciprocals of light intensity, in percent light transmitted are plotted on the abscissa, and reciprocal of the rate of phosphorylation, in μmoles ATP formed per mg chlorophyll per hour, on the ordinate. 100 % Transmission represented about 22,000 ft-c, as measured by a silicon diiode photocell, calibrated against a Weston ft-c meter. Spinach chloroplasts were used, at pH 7, and pyocyanine as a cofactor. Other conditions as described under table I, experiment I.

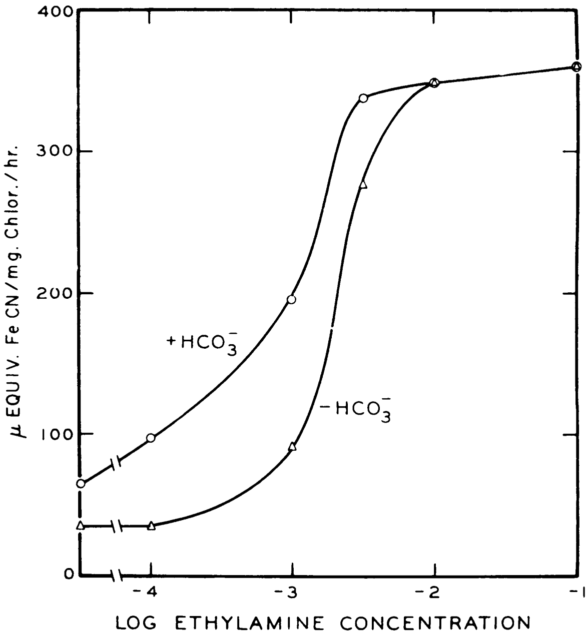

FIG. 2. Nonadditivity of bicarbonate and ethylamine stimulations of the Hill reaction. Bicarbonate, where added, was at a final concentration of 8 mM. Ferricyanide reduction by spinach chloroplasts was measured in a total volume of 3 ml, with ferricyanide at 0.5 mM. Other conditions as in table I.

mum bicarbonate effect is seen only at or below 15 μg of chlorophyll per ml.

We also confirmed, using different chloroplasts and pyocyanine as cofactor, the previous observation (15) that stimulation by added bicarbonate is seen primarily between pH 6.5 and 7.5, i.e., below the optimum pH of 8 for phosphorylation and coupled electron flow. In our earlier experiments this led to some practical difficulties, since only 5 mM dimethylglutarate was in use as a buffer, below the pH optimum. In the control flasks the pH rose as much as 0.4 units during the course of the phosphorylation reaction, thereby raising the rate of phosphorylation slightly. Adding bicarbonate, on the other hand, helped maintain the pH at the lower, suboptimal value, and to that extent tended to depress the rate. Thus, with inadequate buffering, the apparent stimulation by bicarbonate was less than it should have been. Since any correction would only tend to increase the extent of the bicarbonate effect, and since the bicarbonate effect was quite evident even with this handicap, some of the earlier results are included here without correction (table I, Expt I, and table IV). In later work the level of phosphate was raised to at least 10 mM, and this served to maintain a constant pH in all flasks.

The interaction of pH with the bicarbonate effect is reminiscent of the effect of many uncouplers on the pH profile for the Hill reaction (9, 13). It seemed of interest, therefore, to find out whether bicarbonate would continue to stimulate the Hill reaction in the presence of an optimal level of an uncoupling reagent. Figure 2 shows that this is not the case; with enough (10–100 mM) ethylamine added, there is no further effect of added bicarbonate. It should be noted that these experiments were run at pH 7. Since the free base is the effective agent (5, 9), the low pH employed will account for the higher levels of the uncoupler needed to elicit an optimal Hill reaction rate. The ability of bicarbonate to stimulate the Hill reaction was also found to disappear in the presence of optimal concentrations (10^{-4}M) of the uncoupler atebrin (4).

The fact that uncouplers eliminate bicarbonate stimulation stands in contradiction to the observations of other workers. Thus, major CO_2 effects were seen by Good (6) in the presence of either methylamine or DCPIP, and by Stern and Vennesland (18, 19) when trichlorophenol indophenol was used together with ferricyanide. However, in all work previous to that of Punnett and Iyer, a period of CO_2 depletion prior to illumination was needed in order to demonstrate the CO_2 stimulation. Also, CO_2 re-addition was performed previously by flushing with the appropriate gas mixture together with careful pH control. Punnett and Iyer discovered large effects when buffered sodium bicarbonate solution was added to fresh chloroplasts, without worrying about the gradual loss of CO_2 as a gas at pH's from 6.5 to 7.5, since the reactions were performed rapidly. It seemed important, therefore, to investigate the effect of added sodium bicarbonate solution using the methodology of Punnett and Iyer (as in all the experiments so far reported) with CO_2 depleted as well as fresh chloroplasts.

Table II shows results from 2 experiments out of a number performed, both with spinach and pea chloroplasts, and at pH 6.6 and 7.0. Pea chloroplasts were used most routinely, in order to avoid possible artifacts from gradual breakdown of oxalic acid (22) in spinach preparations. It can be seen that a 2-hour incubation under a continuous stream of nitrogen, even without KOH in the center well, can cause the rate of the Hill reaction to fall to 35 to 50 % of its initial value (comparing the rates in the presence of ethylamine). Added bicarbonate restores full activity, and this can represent a 2 to 3.5-fold stimulation. The major point is that the uncoupler cannot replace bicarbonate in this function.

Some other differences between the 2 effects of added bicarbonate are indicated in table III. Under similar conditions, in which bicarbonate is added to the reaction without controlling the gas phase, ferricyanide reduction by depleted chloroplasts responds to concentrations as low as 0.2 mM, and essentially saturates by 2 mM $NaHCO_3$. On the other hand the phosphorylation of fresh chloroplasts (pyocyanine-supported) shows no response until 1 mM, and does not saturate until 8 mM added $NaHCO_3$.

Table II. *Response of the Hill Reaction to Ethylamine and Added Sodium Bicarbonate, before and after CO_2 Depletion*

Reactions were performed aerobically at 20°. The reaction mixture contained 50 mM sodium phosphate at pH 6.6, 200 mM NaCl, 10 mM Na acetate pH 6.6, 0.67 mM potassium ferricyanide, and 75 μg of chlorophyll in 3 ml total volume. These conditions are close to those described by Good (6). CO_2 depletion for 2 hours and measurement of ferricyanide reduction are as described in Methods section. Ethylamine was used at 10 mM final concentration, and sodium bicarbonate at 2 mM. Aliquots from the same preparation of chloroplasts were assayed either before or after CO_2 depletion.

Chloroplasts	Ethylamine	Expt I $-NaHCO_3$	Expt I $+NaHCO_3$	Expt II $-NaHCO_3$	Expt II $+NaHCO_3$
Fresh	−	65*	64	49	66
	+	171	197	288	310
CO_2 depleted	−	68	128	79	73
	+	98	240	85	317

* μequivalents ferricyanide reduced per mg chlorophyll per hour.

Equally important is the fact that pyocyanine supported phosphorylation is not inhibited by the removal of CO_2. In the experiment shown in table III there is even an apparent small stimulation due to CO_2 removal, but this was not usually the case. The response to addition of bicarbonate after CO_2 depletion was extremely variable, ranging from no effect (in this and 3 other experiments) to considerable stimulations, in others.

Punnett and Iyer (15) suggested the possibility that CO_2 might be increasing the efficiency of formation of a high energy intermediate on the phosphorylation pathway. Figure 3 shows that adding sodium bicarbonate decreases the yield of X_E, a high energy state of the chloroplasts defined by the ability to form a limited amount of ATP immediately after illumination (8, 16). Table IV shows that the concentration of bicarbonate needed to inhibit X_E formation (and speed its decay somewhat) are the same as those which do stimulate photophosphorylation, either at pH 6.5 or 7.0. Similar inhibitions of the yield of X_E, with relatively minor effects on the rate of dark decay, were found using either no cofactor, or FMN or ferricyanide in place of the pyocyanine.

Table III. *Comparative Response of Pyocyanine supported Phosphorylation and the Ferricyanide Hill Reaction to CO_2 Depletion and to addition of Bicarbonate at Different Concentrations*

Phosphorylation with fresh and depleted pea chloroplasts were performed on the same day with the same batch of chloroplasts; ferricyanide reductions after CO_2 depletion were taken from a different experiment. Conditions for pyocyanine supported phosphorylation as in table I, using 0.05 μM pyocyanine and 10 μmoles of phosphate in a volume of 3 ml. Ferricyanide reduction was performed in a volume of 3 ml in the presence of 10 mM ethylamine using chloroplasts after CO_2 depletion; all other conditions as in table II.

$NaHCO_3$ conc mM	Hill reaction	Phosphorylation with pyocyanine Fresh	Phosphorylation with pyocyanine CO_2 depleted
0	113*	214**	267
0.2	157	216	248
0.5	177	226	238
2.0	218	265	237
4.0	236	305	279
8.0	241	370	253

* μequivalents ferricyanide reduced per mg chlorophyll per hour.

** μmoles ATP formed per mg chlorophyll per hour.

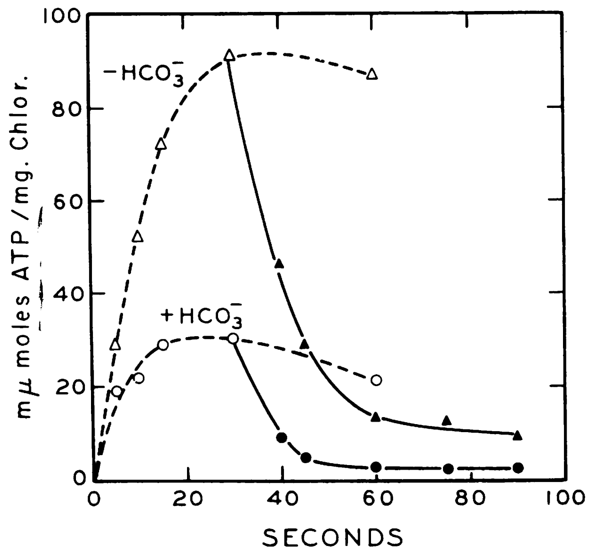

Fig. 3. Time course of X_E formation and decay. Conditions as described under table IV, with pyocyanine and chloroplasts in the light stage at pH 6.5. Calculations of the half lives for decay show 10 seconds in the absence of bicarbonate, and 7 seconds in its presence.

Discussion

Our present data contains 3 major reasons for considering the bicarbonate effect discovered by Punnett and Iyer (15) to be a new one, different from

Table IV. *Effect of Bicarbonate Concentrations on X_E Yield and on Phosphorylation*

For X_E measurement the light stage contained, in 1 ml, pyocyanine at 0.5 mM, Tris-maleate pH 6.5 at 6 mM, 250 µg of chlorophyll and bicarbonate as indicated. The dark stage reaction mixture consisted of an additional 0.5 ml containing, in µmoles: Tris at pH 8, 50; ADP at pH 8, 1.5; $MgCl_2$, 3.0; phosphate at pH 8, 4; and P^{32}. For measurement of phosphorylation pyocyanine was used, with conditions as described under table I, experiment I. The same preparation of spinach chloroplasts was used for both measurements.

HCO_3 conc	X_E yield	Phosphorylation pH 6.5	Phosphorylation pH 7.0
mM			
0	33*	26**	81
2	24	44	113
4	18	58	121
8	16	80	161
16	15	83	166

* mµmoles ATP per mg chlorophyll.

** µmoles ATP formed per mg chlorophyll per hour.

that found by Warburg and Krippahl (22, 23). In the first place the stimulation described by Punnett is not additive with the stimulation caused by optimal amounts of uncoupling agents (fig 2); i.e., the 2 types of reagent seem to replace each other. On the other hand the CO_2 requirement discovered by Warburg not only occurs in the presence of uncouplers, but is maximal when an uncoupler is present (table II and ref. 5).

Secondly, the concentration range of added bicarbonate needed for the 2 effects is somewhat different: that for the Warburg effect lying between 0.2 and 2 mM, and that for the Punnett effect between 1 and 8 mM (table III and ref. 13). Since the composition of the gas phase was not controlled in the present experiments (nor in those of Punnett and Iyer) it cannot be claimed that these are the absolute concentrations present in the reaction mixtures. However the addition of bicarbonate, pH of the reaction mixture and subsequent time of illumination was the same for both types of experiments in the present work. A relative comparison of concentration requirements is therefore valid.

The third major point of difference is that the Punnett effect clearly applies to phosphorylation supported by a pyocyanine-dependent cyclic electron flow (table II). On the other hand, the Warburg CO_2 requirement cannot be demonstrated with this type of phosphorylation (table III). These last data indicate that the CO_2 requirement discovered by Warburg does indeed apply primarily to a site related to the mechanism of O_2 evolution, as originally postulated (22, 23). The Punnett effect, on the other hand, is independent of O_2 evolution and probably functions at some point in the phosphorylation pathway, again as originally postulated (15).

It is clear that the Warburg CO_2 effect can be called a requirement, since the rate is depressed by removal and restored by adding back the CO_2 or bicarbonate. An interesting point is that CO_2 depletion does not depress the basal rate of the Hill reaction under our present conditions, although it did so in the manometric experiments of Good (6). Although our results might suggest a difference in the nature of the basal from the uncoupled electron transport, they could just as easily result from a failure to remove CO_2 completely. The remaining traces of the gas might be sufficient to support the low rate basal Hill reaction, which is in any event already limited by a combination of the phosphorylation mechanism and the low pH. In this case we could infer that CO_2 functions at some other point, not at the limiting reactions of the coupling mechanism.

On the other hand, the effect discovered by Punnet and Iyer should be called a stimulation, not a requirement. Removal of CO_2 did not inhibit phosphorylation and cyclic electron transport (table III) although these were stimulated by added high levels of bicarbonate. Bicarbonate in this case probably causes a release of the inhibition brought on by the low pH, below the ordinary optimum. At the pH optimum adding bicarbonate causes no further stimulation (15). Consistent with this role is the fact that uncouplers, which in their own right cause a release of the low pH inhibition of the Hill reaction, also eliminate any observable effect of added bicarbonate (fig 2). However, saying that bicarbonate overcomes the inhibition due to a low pH does not explain its mode of action in this case. Also we would like to emphasize that no other reagent has been discovered, other than the basic components of the reaction mixture, which will stimulate photophosphorylation, in this or any other pH range.

Punnett and Iyer suggested that dissolved CO_2 was the effective molecular species because their calculations showed its concentration was roughly the same in theirs and in all the previous studies. However we have shown here that the effect demonstrated by Punnett and Iyer is probably a new one, and it is hard to see why the same concentration of dissolved CO_2 could have a different effect due to the method of addition. We can speculate, therefore, that bicarbonate may be needed for the Punnett and Iyer effect, rather than dissolved CO_2.

We find it difficult to rationalize the fact that added bicarbonate can stimulate the basal rate of the Hill reaction, in the absence of either uncouplers or ADP and phosphate (fig 2 and ref. 14). Since bicarbonate raises the P/2e ratio and makes phosphorylation more efficient in this low pH range, one might expect it to cause tighter coupling and therefore depress the basal rate. Looking at it another way, as far as its effect on the rate of electron transport is concerned bicarbonate acts as an uncoupler would; but when it comes to the phosphorylation reaction proper it stimulates instead of inhibiting.

Punnett and Iyer made the very attractive suggestion that bicarbonate may increase the efficiency of formation of some high energy intermediate of the phosphorylation pathway (14). Previously we had considered X_E to be such an intermediate; and the fact that bicarbonate depresses its steady state level and causes a slightly faster breakdown (fig 3) would be disconcerting from that point of view. However, more recent evidence as to the large amount and slow kinetics for formation of X_E (11, 16) suggests to us that X_E as ordinarily measured may be on a side pathway, storing energy derived from true intermediates of the phosphorylation mechanism in a reservoir as an alternative to ATP (possibly as a transmembrane hydrogen ion gradient; see ref. 14). In that case, formation and turnover of X_E would be a drain on the direct pathway to ATP. It may be that bicarbonate functions by shutting off, to some extent, access to this side reservoir otherwise favored by the low pH; and makes the direct pathway to phosphorylation more efficient in this manner.

Summary

We have repeated both the stimulation of phosphorylation and the Hill reaction by added bicarbonate as discovered by Punnett and Iyer, and the carbon dioxide requirement for the Hill reaction as discovered by Warburg and Krippahl. Additional evidence presented here suggests that these are 2 distinct effects. The Warburg carbon dioxide requirement pertains to electron transport at a site close to the evolution of oxygen; whereas added bicarbonate as used by Punnett probably acts on a part of the phosphorylation mechanism inhibited by a suboptimal pH.

At a concentration sufficient to stimulate photophosphorylation, bicarbonate inhibits formation of a nonphosphorylated, high energy condition of chloroplasts (X_E). If X_E represents a side pathway draining energy from the phosphorylation mechanism, this could be the basis for the increase in phosphorylation efficiency caused by high levels of bicarbonate added to fresh chloroplasts.

Acknowledgment

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