Plant Physiol. (1975) 55, 515–519

Carbon Dioxide and Senescence in Cotton Plants

Received for publication July 18, 1974 and in revised form October 30, 1974

CHONG W. CHANG
Western Cotton Research Laboratory, United States Department of Agriculture, Phoenix, Arizona 85040

ABSTRACT

Glandless cotton plants (*Gossypium hirsutum* L. cv. Coker 100) were subjected to the influence of high CO_2-bicarbonate. The content of protein decreased with no accompanying increase in its degraded products. The decrease in protein was correlated with the low content of chlorophyll and also with the reduced activity of carbonic anhydrase. The initiation of these correlations coincided with the time when the control leaves contained the highest enzyme activity during leaf growth. The high concentration of bicarbonate directly restricted the rate of photophosphorylation and that of the Hill reaction in isolated chloroplasts. The amount of ATP in leaves treated *in vivo* also diminished. High CO_2 as bicarbonate, however, did not directly inhibit the activity of carbonic anhydrase *in vitro*.

The average CO_2 content of the atmosphere is about 300 μl/l. With kohlrabi and spinach chloroplast preparations, Warburg and Krippahl (15) and Abeles *et al.* (1), respectively, reported that high concentrations of CO_2 ranging from 15×10^3 to 50×10^3 μl/l stimulated the rate of the Hill reaction. With oat chloroplast suspensions, Punnett and Iyer (13) also demonstrated that high concentrations of CO_2, 2 to 10 mM as bicarbonate, increased the rates of photophosphorylation and the Hill reaction. Carbonic anhydrase may play an essential role in CO_2 assimilation (4, 5). Graham and Reed (6) showed that carbonic anhydrase regulates photosynthesis of *Chlorella* at low levels of CO_2.

Experiments conducted in my laboratory revealed that whole cotton plants treated with 850 to 1000 μl/l CO_2 in controlled environment chambers senesce gradually during a growth period of about 30 days. This adverse symptom could not be explained by the previous reports of stimulatory effects of high CO_2 on the rates of photophosphorylation and the Hill reaction (1, 13, 15), or the claimed beneficial and regulatory roles of carbonic anhydrase in CO_2 assimilation (4–6).

The reaction of CO_2, when hydrated in leaf tissue at physiological pH values of about 6, would be: $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$. Hydration of CO_2 and dehydration of HCO_3^- in these reactions are catalyzed by carbonic anhydrase. Since CO_2 and HCO_3^- are substrates for this enzyme, an investigation into the possible relationships of carbonic anhydrase with reactions linked to CO_2 assimilation and with other related changes has been pursued under the influence of high CO_2.

The goals of the present work were (*a*) to study responses of the activity of carbonic anhydrase to various concentrations of CO_2 and (*b*) to elucidate the relationships between this enzyme activity and other metabolic alterations associated with senescence in cotton leaves caused by high concentrations of CO_2.

MATERIALS AND METHODS

Glandless cotton plants (*Gossypium hirsutum* L. cv. Coker 100) were raised in a growth chamber in a sand-peat moss-vermiculite mixture and watered with nutrient solution. Temperature was about 32 C during the day and about 22 C at night. A 15-hr photoperiod was maintained with a light intensity of 28.5 klux. Relative humidity was about 75% during the day. The treatment of plants with CO_2 (99.9 min volume per cent in purity) was conducted in two controlled environment chambers, one for control and the other for elevated CO_2 levels, where concentrations of 650, 850, or 1000 μl/l were maintained. The gas from a compressed CO_2 tank kept at room temperature was passed into the chambers. The levels of CO_2 in the control and treatment chambers were determined by continuous monitoring with an infrared gas analyzer.

Cotton leaf laminae (4.5 g) were homogenized at 4 C in 50 ml of 0.1 M tris-HCl buffer, pH 8.3, containing 3 mM EDTA and 0.03 M 2-mercaptoethanol. The homogenization was conducted three times in the Omni-Mixer. A short stop (20 sec) after each 20 sec was made. The material was squeezed through two layers of cheesecloth, and the filtrate was centrifuged at 2700*g* for 5 min. The supernatant fraction was decanted. The residue was made into a thin paste by pressing the wet material against the inside wall of the centrifuge tube with the round end of a glass test tube. To this sample the supernatant solution was added. The mixture was stirred gently to obtain a uniform suspension. These processes were found to be necessary to remove the foaming which tended to occur in the fresh filtrate and interfered with enzyme assays.

Carbonic anhydrase was assayed by the electrometric methods of Wilbur and Anderson (16), described by Chen *et al.* (4) with minor modifications. Two milliliters of 0.02 M veronal buffer at pH 8 and 1 ml of enzyme extract were pipetted into a vial (2.5 × 7 cm), embedded in ice. CO_2-saturated H_2O (3.5 ml) was added rapidly from a wide mouth pipette. The CO_2-saturated substrate was made by bubbling the gas from a compressed CO_2 tank into ice-cold H_2O for 30 min. The pH change was then monitored with a pH meter equipped with a glass electrode. The time in seconds for the pH to drop to 6.3 due to enzyme activity was designated as T (20–24 sec with the sample preparation from the leaves at the most active age). The time taken for a similar drop by an enzyme extract boiled for 5 min was designated as T_o (130–140 sec). Activity units were expressed as $10(T_o/T\text{-}1)$/mg Chl or g fresh weight leaves/sec. An average of two determinations was made for each activity. However, if the difference in replicate readings was more than 5 sec, an additional assay was conducted. The crude enzyme preparation when treated with insoluble PVP did not change in activity.

For determining effects of bicarbonate on the rate of photophosphorylation, the procedure of Arnon *et al.* (3) was followed except for the following modifications. The reaction was carried out aerobically in Gilson respirometer vessels. The

main compartment contained (*a*) 80 μmoles of tris-HCl buffer, pH 8 (or 7.5), (*b*) 5 μmoles of $MgCl_2$, (*c*) 10 μmoles of ADP, (*d*) 0.1 μmole of phenazine methosulfate, and (*e*) various concentrations of $KHCO_3$ and chloroplasts containing 0.2 mg of Chl in a final volume of 3 ml. ADP was neutralized to pH 6.6 immediately prior to use. The side arm contained 10 μmoles of K_2HPO_4. The chilled vessels were attached to the respirometer and shaken at 15 C for 10 min in the dark. The reaction was started by pouring the contents of the side arm into the main compartment and was carried out with shaking under illumination (18 klux). After 30 min, the reaction was terminated by turning off the light. The reaction mixture was immediately transferred to a centrifuge tube under a dim green light and spun at 35,000*g* for 10 min. The ATP concentration in the supernatant was determined with luciferase (7). For determining the effects of bicarbonate on the rate of the Hill reaction, the procedure of Jagendorf and Evans (9) was followed with slight modifications. The vessel contained 72 nmoles of 2,3,6-trichloroindophenol, 150 μmoles of tris-HCl buffer, pH 8 (or 7.5), various concentrations of $KHCO_3$, and chloroplasts containing 0.03 mg of Chl in a total volume of 3 ml. The reaction mixture was incubated for 60 sec under illumination (18 klux) in a shaking water bath at room temperature. Light intensity was saturating, since the rate was directly related to the Chl concentration. One unit of activity is defined as the change in absorbance/min at 620 nm. Each experiment was corrected for the apparent phosphorylation or reduction occurring in a dark control. Chloroplasts were prepared in buffer at pH 8 and suspended in buffer at pH 7.5 according to the procedure of Jagendorf and Evans (9).

Acid-soluble nucleotides and ATP were determined by the procedure of Guinn (8), and that of Guinn and Eidenbock (7), respectively. Ninhydrin-positive compounds were extracted with 70% (v/v) ethyl alcohol. Alcohol was removed under vacuum. Chlorophyll associated with the sample was separated by shaking with chloroform, and the aqueous layer was removed by pipetting after centrifugation. A fraction of sample was assayed for ninhydrin-positive compounds by the method of Moore and Stein (11). The content of Chl was determined by the method of Arnon (2). The amount of protein was determined by the procedure of Ma and Zuazaga (10). All determinations were done in duplicate or triplicate.

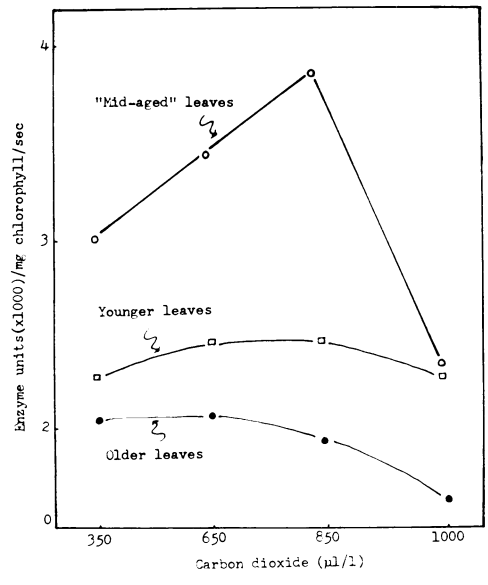

FIG. 1. Effect of various concentrations of CO_2 on activities of carbonic anhydrase (per mg Chl per sec) in leaves at three different ages. Leaves of cotton plants (22 days from germination) were exposed to various concentrations of CO_2 for 30 days and leaves were sampled every 5 days. An average enzyme activity of the first two samples (5 and 10 days old), that of the last two samples (25 and 30 days old), and the activity of leaves 15 days old were designated as values of enzyme activity from "younger" (□), "older" (●), and "mid-aged" leaves (○), respectively. The responses of enzyme activity in similar "younger", "older", or "mid-aged" leaves to different concentrations of CO_2 were determined. Control leaves were exposed to the ambient level of CO_2, about 350 μl/l.

RESULTS AND DISCUSSION

Figure 1 shows that carbonic anhydrase activities in the "younger" leaves did not deviate much from the control at any CO_2 level. In the "mid-aged" leaves the changes in activities of carbonic anhydrase rose linearly, proportional to the concentrations of CO_2 up to 850 μl/l, and then diminished in activity by 1000 μl/l CO_2. However, this curve changed, when the "mid-aged" leaves remaining became older and were exposed to ensuing CO_2 treatment. These "older" leaves showed a progressive reduction in enzyme activity with increasing concentrations of CO_2 after a lag period of enzyme activity at the lowest level of CO_2 (650 μl/l). The decrease in enzyme activity began when leaves subjected to 850 or 1000 μl/l CO_2 were approximately 25 or 15 days old, respectively. Thus, the enzyme probably could not maintain its proper level of activity in CO_2 assimilation. This finding was in contrast with the report (6) showing the regulatory role of carbonic anhydrase in photosynthesis at low levels of CO_2.

Figure 2 shows that the curve of the control protein content increased with leaf age up to the eighth node. Thereafter the amount of protein decreased sharply (upper right, Fig. 2). This curve paralleled that of enzyme activities at various levels of leaf age (upper left, Fig. 2) except for the higher protein content in mature leaves. This higher protein level appeared to result from a formation of structural protein accompanied by tissue differentiation at the later stages of leaf development. High CO_2 caused the protein decline in leaves of various ages. These changes in protein content were also closely related to changes in enzyme activity (lower, Fig. 2). The decreases in both protein and ATP (Table I), together with no accompanying increase in ninhydrin positive compounds (Table I) provide evidence that high CO_2 decreases the content of protein, not by degradation, but by curtailing protein formation.

Figure 3 shows that the most active preparation of enzyme activity came from leaves about 2 weeks old in control plants. A level of 1000 μl/l CO_2 drastically decreased this peak level of enzyme activity (upper, Fig. 3). In leaves treated with 1000 μl/l CO_2, the relative content of Chl began to decrease, and the enzyme activity also began to drop from the control level at a leaf age of about 10 days. Thereafter, this reduction in Chl paralleled the course of the declining activity caused by high CO_2. To assess the adverse effects of high CO_2 on the total activity of carbonic anhydrase and the level of senescence, the leaves of entire plants were analyzed for activity and Chl content. Figure 4 shows that cotton leaves treated with 1000 μl/l CO_2 for 30 days lost significant enzyme activity and Chl per unit leaf area, whereas only about a 12% decrease occurred in growth (inset, upper right, Fig. 4).

From the data shown in Figures 2, 3, and 4, it was obvious that the decrease in protein content was closely related to the reduction in the activity of carbonic anhydrase and also to the decline in Chl in leaves exposed to high CO_2. The relationship between the decline in both protein and Chl contents is a well known phenomenon linked with senescence (14).

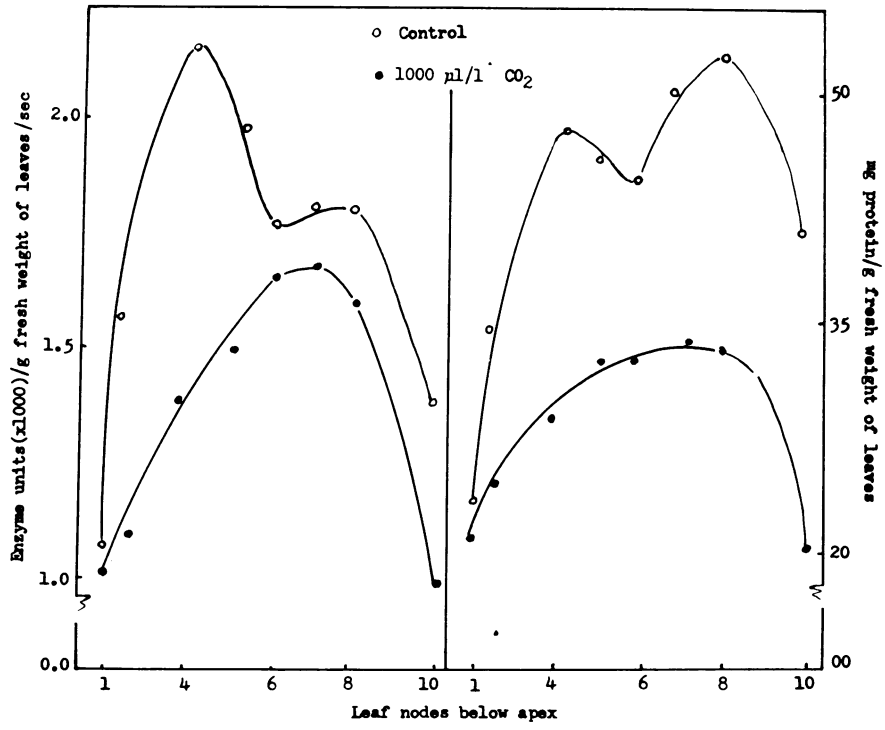

FIG. 2. Protein content and carbonic anhydrase activity in leaves exposed to 1000 µl/l CO_2 for 28 days. Leaves were sampled at the various nodes below the apex. The activity of enzyme and the content of protein (per g of fresh leaves) were calculated by multiplying the enzyme units and protein content per mg Chl by the total Chl content previously determined in a known amount of fresh leaves.

Table I. *Effect of CO_2 on Content of Ninhydrin Reactive Compounds, Acid-soluble Nucleotides, and ATP Determined in Cotton Leaves*

The leaves were about 2 weeks old from the time when the leaves (about 2 cm in diameter) were exposed to 1000 µl/l CO_2. Each value was an average of three determinations. Their coefficients of variation $\left(CV = \frac{100S}{\bar{X}}\right)$ fell between 4 and 6. The data were consistent with those from repeated experiments.

Compounds	Control	1000 µl/l	Percentage of control
	µmoles/g dry wt leaves		
Ninhydrin reactive compounds as glycine	81.2	50.3	62
Acid-soluble nucleotides as AMP	37.9	22.4	59
ATP	0.32	0.14	43

Figure 5 (lower, left) shows the effects of various concentrations of bicarbonate on the rate of photophosphorylation in chloroplast suspensions. Luciferase was used to estimate the level of ATP formed as described under "Materials and Methods." The capacity of this enzyme to react with ATP was satisfactory without an interference with increasing concentrations of bicarbonate and other components in the reaction mixture. A known amount of ATP (1 µg), instead of ADP, added to the reaction mixture was quantitatively determined as shown by the horizontal line. At pH 7.5, all tested concentrations of bicarbonate inhibited photophosphorylation. However, at pH 8 the rate was stimulated in an extremely narrow concentration range of bicarbonate (below about 1 mM) and

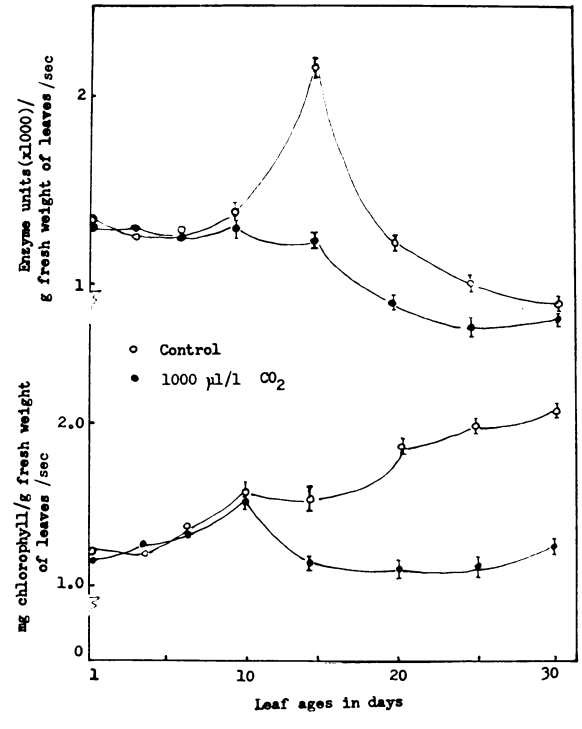

FIG. 3. Activity of carbonic anhydrase (upper) and the content of Chl (lower) in developing cotton leaves subjected to 1000 µl/l CO_2 for 28 days. Leaf ages shown on the abscissa were days counted from the time when the youngest leaves (conveniently 1 day old) were about 2 × 2 cm. The distance between the horizontal bars at each point indicates the standard error.

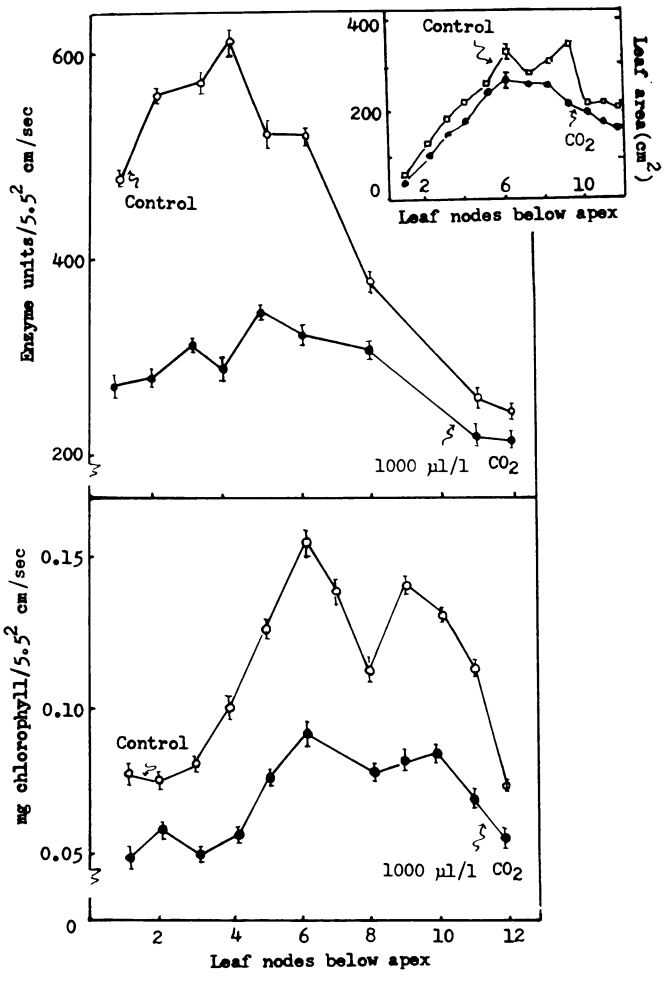

FIG. 4. Activity of carbonic anhydrase and the content of Chl per unit area (5.5² cm) of leaves of various ages under the influence of 1000 µl/l CO_2 for 30 days. The figure in the inset shows the growth rates of leaves as determined by multiplying leaf length by leaf width. The distance between the horizontal bars at each point indicates the standard error.

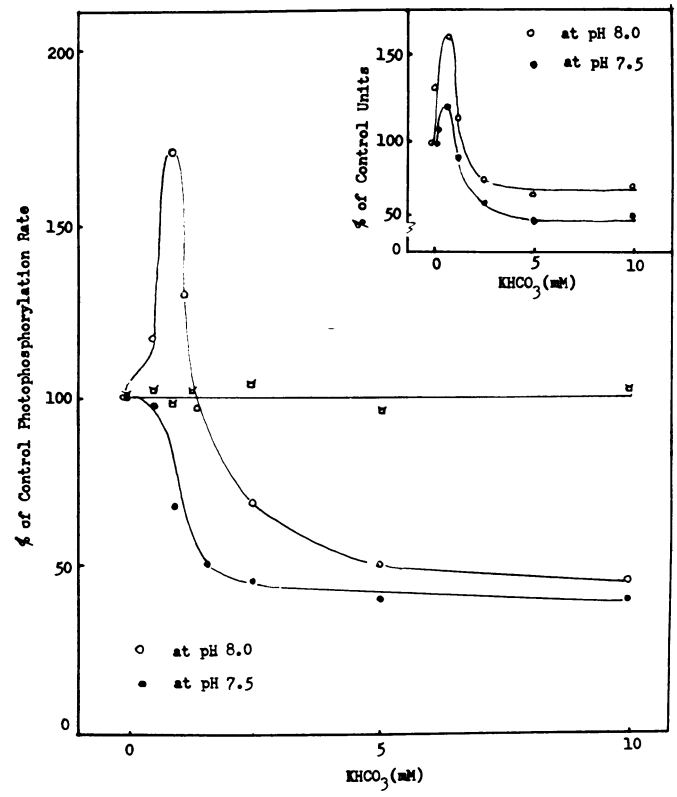

FIG. 5. Effect of various concentrations of bicarbonate on the rate of photophosphorylation (lower, left) and on the rate of the Hill reaction (upper, right). Each value was an average of three determinations. For determining the level of ATP by measuring luminescence, 0.2 ml of sample were injected into the light-tight cuvet containing 1 ml of 0.05 M glycine buffer at pH 7.4 and 0.2 ml of crude luciferase (7). This was repeated for each determination. The values were then averaged. The control activities of photophosphorylation and the Hill reaction were 33 µmoles ATP/hr·mg Chl and 12.4 units/mg Chl, respectively. One unit of activity in the Hill reaction was defined as change in absorbance per min at 620 nm.

diminished sharply by a further increase in this anion. These data seemed to differ from the observations of photophosphorylation in oat (13) and Chromatium strain D chloroplasts (12), which were stimulated by much wider ranges of bicarbonate concentrations, 1 to 7 mM and up to about 50 mM, respectively. Such sensitive properties of cotton chloroplasts to bicarbonate appear to be associated with the senescence caused by high CO_2. Activity of the Hill reaction responded to bicarbonate similarly to photophosphorylation (inset, upper right, Fig. 5).

The reduction in the rate of photophosphorylation of cotton chloroplasts by high bicarbonate *in vitro* (Fig. 5) was consistent with the decrease in ATP content of leaves in high CO_2 (Table I), which was also related to the lowered amount of total nucleotides (Table I). The data indicate that the content of ATP decreased, not by the conversion of this compound to other species of nucleotides, but by a lowered rate of formation.

The effect of CO_2 (as bicarbonate) on the activity of isolated carbonic anhydrase *in vitro* was also investigated. The curve for the enzyme velocity followed the general pattern of the effect of increasing substrate concentrations on the rate of enzyme action *in vitro*. The maximal velocity of enzyme activity, following a linear increase, was independent of further increase in the substrate concentration. These data demonstrate that high CO_2 does not directly inhibit the activity of carbonic anhydrase from cotton leaves. Also the decreases in the activity of carbonic anhydrase and in such constituents as Chl and protein coincided with the time (about 2 weeks or fourth node) when the control leaves contained the highest enzyme activity during leaf growth. These relationships show that the catalytic hydration of CO_2 into bicarbonate by the peak activity of carbonic anhydrase seemed to precede causally the initiation of senescence. If this assumption is correct, carbonic anhydrase activity in high CO_2 is likely to be adverse rather than beneficial.

LITERATURE CITED

1. ABELES, F. B., A. H. BROWN, AND B. C. MAYNE. 1961. Stimulation of the Hill reaction by carbon dioxide. Plant Physiol. 36: 202-207.
2. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1-15.
3. ARNON, D. I., F. R. WHATLEY, AND M. B. ALLEN. 1964. Photosynthesis by isolated chloroplasts. II. Photosynthetic phosphorylation, the conversion of light into phosphate bond energy. J. Amer. Chem. Soc. 76: 6324-6328.
4. CHEN, T. M., R. H. BROWN, AND C. C. BLACK. 1970. CO_2 compensation concentration, rate of photosynthesis, and carbonic anhydrase activity of plants. Weed Sci. 18: 399-402.

5. Everson, R. G. 1970. Carbonic anhydrase and CO_2 fixation in isolated chloroplasts. Phytochemistry 9: 25–32.
6. Graham, D. and M. L. Reed. 1971. Carbonic anhydrase and the regulation of photosynthesis. Nature New Biol. 231: 81–82.
7. Guinn, G. and M. P. Eidenbock. 1972. Extraction, purification, and estimation of ATP from leaves, floral buds, and immature fruits of cotton. Anal. Biochem. 50: 89–97.
8. Guinn, G. 1973. Purification of leaf nucleotides and nucleosides on insoluble polyvinylpyrrolidone. Anal. Biochem. 54: 276–282.
9. Jagendorf, A. T. and M. Evans. 1957. The Hill reaction of red kidney bean chloroplasts. Plant Physiol. 32: 435–440.
10. Ma, T. S. and G. Zuazaga. 1942. Micro-Kjeldahl determination of nitrogen. A new indicator and improved rapid method. Ind. Eng. Chem. 14: 280–282.
11. Moore, S. and W. H. Stein. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. 176: 367–388.
12. Murai, T. and T. Akazawa. 1972. Bicarbonate effect on the photophosphorylation catalyzed by chromatophores isolated from Chromatium strain D. Plant Physiol. 50: 568–571.
13. Punnett, G. and R. V. Iyer. 1964. The enhancement of photophosphorylation and the Hill reaction by carbon dioxide. J. Biol. Chem. 239: 2335–2339.
14. Srivastava, B. I. S. 1967. Cytokinins in plants. Int. Rev. Cytol. 22: 349–387.
15. Warburg, O. and G. Krippahl. 1958. Hill reaction. Z. Naturforsch. 136: 509–514.
16. Wilbur, K. M. and N. C. Anderson. 1948. Electrometric and colorimetric determination of carbonic anhydrase. J. Biol. Chem. 176: 147–154.