Influence of pH upon the Warburg Effect in Isolated Intact Spinach Chloroplasts

I. CARBON DIOXIDE PHOTOOASSIMILATION AND GLYCOLATE SYNTHESIS

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ABSTRACT

The influence of pH upon the O2 inhibition of 14CO2 photoassimilation (Warburg effect) was examined in intact spinach (Spinacia oleracea) chloroplasts. With conditions which favored the Warburg effect, i.e. rate-limiting CO2 and 100% O2, O2 inhibition was greater at pH 8.4 to 8.5 than at pH 7.5 to 7.8. At pH 8.5, as compared with 7.8, there was an enhanced 14C-labeling of glycolate, and a decrease of isotope in some phosphorylated Calvin cycle intermediates, particularly triose-phosphate. The 14C-labeling of starch was also more inhibited by O2 at higher pH. The enhanced synthesis of glycolate during 14CO2 assimilation at higher pH resulted in a diminution in the level of phosphorylated intermediates of the Calvin cycle, and this was apparently a causal factor of the increased severity of the Warburg effect.

The 14C-labeling profiles have been interpreted in terms of a "CO2"-sensitive as well as a "CO2"-insensitive mechanism for glycolate synthesis. Both mechanisms functioned optimally at the higher pH and both responded to O2.

Many hypotheses have been proposed to explain the Warburg effect or the inhibition of CO2 photoassimilation by O2 (7, 17). One popular explanation is based upon an enhanced synthesis of glycolate in response to O2 resulting in a diminution of ribulose-1,5-diphosphate generation (2, 3, 5). The consequence would be a decreased CO2 uptake. However, attention has been addressed in both whole plants and isolated chloroplasts to glycolate yield and RuDP generation in terms of the concentration of CO2 and O2. It has been well established that glycolate levels are directly related to O2 but inversely to CO2 (5, 7, 15, 19).

There are recently published reports indicating that the pH of the reaction solution must also be considered a factor in glycolate yields. During photosynthesis under optimal conditions, i.e. saturating levels of CO2 in air, pH 8.5 in contrast to 7.5 increase glycolate yields in intact algal cells (4, 11) and in algal as well as spinach chloroplasts (4, 10). In all of these investigations, the effect of pH was monitored at only one concentration of CO2 and O2. The purpose of the present investigation is not only to evaluate the effect of pH on glycolate yield when the CO2 and O2 concentrations were varied, but also to examine the influence of pH of the reaction mixture upon the Warburg effect in the isolated intact spinach chloroplast. A preliminary account of this investigation has appeared (16).

MATERIALS AND METHODS

Plant Material. Spinacia oleracea was propagated in a controlled environment chamber as previously indicated (15). Additionally, spinach plants were propagated from seed in a 66:34 mixture of sterile commercial potting soil-Terralite vermiculite, irrigated with commercial Rapid-Gro fertilizer solution (weekly at approximately 3.5 g/l), and allowed to mature in the greenhouse (September to May). Varieties of S. oleracea used included Virginia Savoy, Winter Bloomsdale, and Bloomsdale Long Standing. All seed lots were purchased from Agway, Inc.

Intact Chloroplast Isolation. Intact chloroplasts were prepared from 5 to 10 g of deveined spinach leaf tissue and Chl was measured by previously published procedures (8). The pellet which resulted from the final centrifugation, and which contained intact chloroplasts, was resuspended in 2 to 3 ml of the homogenizing medium (at 5°C). This preparation contained 40 to 100 μg Chl/0.10 ml. The homogenizing-resuspending medium contained 0.05 M HEPES (pH 6.8), 0.33 mM sorbitol, 1 mM Na4P2O7, 2 mM EDTA, 1 mM MgCl2, and 1 mM MnCl2.

Plastid 14CO2 Assimilation. Light-dependent 14CO2 assimilation was carried out at 25°C in 1- to 2-ml reaction mixtures containing 0.05 M HEPES at pH 7.5 to pH 8.4, or 0.05 M Tricine at pH 8.4 to pH 8.5, 0.33 mM sorbitol, 1 mM Na4P2O7, 2 mM EDTA, 1 mM MgCl2, and 1 mM MnCl2. The mixtures were brought to the appropriate pH with 12N NaOH. The mixtures contained 30 to 50 μg chloroplast Chl/ml. The effects of O2 and pH upon chloroplast photosynthesis were identical regardless of whether reaction mixtures were buffered with HEPES or Tricine buffer. The pH of each mixture was established by inserting a pH microelectrode unit into the complete reaction mixtures including plastids. The reaction mixtures contained 0.47 μM to 5.47 μM total "CO2" and 40 μCi 14C/mM. The concentration of dissolved CO2 in the reaction mixtures was computed as a function of pH at 25°C employing the rate constants for CO2 hydration and HCO3- dehydration reported by Gibbons and Edsall (6).

The reaction mixtures were aerated before and during the plastid photosynthesis period with 100% O2 (approximately 1 mM in solution), 21% O2 (approximately 0.25 mM O2 in solution), or 100% N2. The gas streams were passed through water and soda lime filters prior to their entrance into the reaction solutions. The gases were passed into the reaction mixtures through plastic straws.

Photosynthetic 14CO2 assimilation was assayed in a system...
which was previously described (15) with saturating incident light intensity from one side of the reaction tube measured to have a value of 1050 w/m². Aliquots of 0.1 to 0.2 ml were taken from the mixtures as a function of time increments, instantaneously added to 20 µl of 12 n HCl, and the resulting acid-stable, total 14C-labeled intermediates were measured with gas flow-end window detection as previously described (15). Methods for computation of 14CO2 assimilation kinetic data employed have been reported previously (8).

**Products of Photosynthesis.** Acid-stable, 14C-labeled intermediates synthesized after 10 to 12 min of assimilation were separated and identified employing one-dimensional descending paper chromatography on Whatman No. 1 paper. The chromatograms were developed one dimensionally using Wood's GW, solvent (15); autoradiography of chromatograms and other techniques used for product identification were similar to those previously reported from this laboratory (15). Except for 14C-glycolate, excised areas of the chromatogram containing the products or groups of products were placed in scintillation vials with 15 ml of fluid containing 5 g PPO/l toluene. The radioactivity in each product group of products was determined utilizing a Beckman LS-250 scintillation counter. Carbon 14-labeled glycolate was separated and purified employing Dowex-acetate chromatography. The radioactivity in the purified glycolate fraction was compared and normalized with that radioactivity in the paper chromatogram fractions associated with all other 14C-labeled products. All product fractions represented the total 14C incorporated in an aliquot of reaction mixture after 10 to 12 min of photosynthesis. The isolation of 14C-glycolate was carried out in essentially the same manner as previously described (15) with some exceptions. The insoluble material was removed by centrifugation from a 0.6-ml aliquot of the reaction mixture treated with 12 n HCl and the supernatant fraction was quantitatively combined with 30 to 50 ml of deionized-distilled H2O, and finally brought to pH 7 with 12 n NaOH. This preparation was passed across a Dowex AG 1-8X acetate (100–200 mesh) column (0.8 x 7 cm resin bed) at the flow rate of 1 ml/min to allow complete adsorption of 14C products. The column was then washed with 50 ml of deionized distilled H2O. When the wash was complete, 4 n acetic acid was added to the resin bed, and elution was begun at a rate of 1 ml/min. Elution was followed until the pH of the eluate was 3, and then 1 ml was collected and discarded. The next 16 ml of 4 n acetic acid contained all of the 14C-glycolate. Radioactivity in the eluate samples was determined by placing 0.10 ml of the Dowex-acetate eluate with 8 ml of scintillation fluid which contained 5 g PPO and 100 g naphthalene/dioxane. The total 14C incorporated into all acid-stable products was determined for the aliquot of 14CO2 assimilate fractionated for 14C-glycolate, and the 14C-glycolate to 14C total photosynthetic ratio was computed.

Known amounts of 14C-glycolate were added to equivalent reaction mixtures containing chloroplasts, these reaction mixtures were acidified with 12 n HCl, and the isolation steps above were carried out. Recovery of known amounts of 14C-glycolate from these mixtures was 95 to 100%, and recovery controls were carried out with each experiment.

In order to establish that only 14C-glycolate was present in the 4 n acetic acid eluates from Dowex-acetate chromatography, aliquots of the eluates were subjected to one-dimensional paper chromatography employing a solvent system described by Zelitch (18). The solvent contained 1-butanol-95% ethanol-H2O-diethylylamine in the proportions 80:10:20:1. Each chromatogram was developed twice in the same direction (consecutively) in this solvent, and since the fractions could also contain 14C-glycerate, unknowns were co-chromatographed with known amounts of standard 14C-glycerate as well as 14C-glycolate. X-ray film autoradiography was employed to identify the labeled products. Regardless of the conditions present in the chloroplast reaction mixtures, the 4 n acetic acid eluates were found to contain only 14C-glycolate.

**Chloroplast Integrity and Reaction Mixture Aeration.** To estimate the effect of O2 aeration upon chloroplast integrity at pH 7.5 compared with pH 8.5, we measured the activities of RuDP carboxylase inside and outside the chloroplast, prior to and after O2 or N2 aeration. RuDP carboxylase was assayed according to a published procedure (1).

Intact plastid preparations were incubated in the sorbitol medium with reaction mixtures at pH 7.5 or pH 8.5. Alternatively, chloroplast preparations were incubated in a medium identical to the sorbitol-containing mixtures except that sorbitol was omitted to facilitate osmotic disruption of intact plastids. With "CO2" excluded from these plastid mixtures, they were illuminated as well as aerated for 15 min with streams of 100% O2 or N2. At the end of this period, illumination and aeration were terminated, and 5.7 µmol (40 µCi) of 14CO2, 20 µmol MgCl2, and 4 µmol dithioerythritol were supplied to the reaction mixture (11.1 ml).

After a 5-min incubation period, 0.50 µmol RuDP was supplied. RuDP carboxylase activity was also estimated in chloroplast preparations which had not been aerated, but which had been incubated with and without sorbitol in the reaction mixture.

Initially, and without aeration, 70 to 80% of the RuDP carboxylase activity was associated with the plastid and the remaining 20 to 30% was outside the chloroplasts. This distribution was independent of pH. After 15 min aeration with O2 or N2, and regardless of pH, approximately 40 to 48% of the total RuDP carboxylase remained inside the chloroplasts. Thus, approximately 40 to 50% of the chloroplasts were disrupted as a result of aeration independent of O2 and pH.

**RESULTS**

**Influence of pH upon O2 Inhibition of CO2 Assimilation.** When the total "CO2" (0.68 mm) in the reaction mixture was rate-limiting, and the reaction mixtures were treated with a stream of 100% O2, O2 in contrast to N2 inhibited CO2 fixation 22.1% at pH 7.55 and 84.2% at pH 8.4 (Fig. 1). When the reaction medium was aerated with 21% O2 (or N2), photosynthesis was inhibited approximately 14 to 30% at pH 7.5 and 73% at pH 8.4 (data not shown). When the total "CO2" in solution was 0.68 mm, the CO2 present at equilibrium was computed to be 27 µmol at pH 7.55 and 3.9 µmol at pH 8.4.

A feature of the O2 inhibition of photosynthesis is its reversibility associated with the lowering of the O2 concentration in the chloroplast-containing reaction mixture during the course of photosynthesis (5). In experiments similar to those just described, it was observed that the pH of the reaction medium influenced the degree of reversibility. When the reaction mixture pH was 7.8 (and CO2 was rate-limiting), the replacement by N2 aeration after 6 min of O2 aeration during the photosynthesis reaction period (about 46% O2 inhibition) resulted in immediate and complete relief of the O2 inhibition. With the reaction mixture pH at 8.5, and after 6 min of aeration with 100% O2 during the initial photosynthesis period, the O2 inhibition of plastid photosynthesis (78%) could only be partially relieved (65%) even after 12 additional min of N2 aeration.

In reference to the limited reversibility of the O2 inhibition at alkaline pH intact chloroplasts are not disrupted as a result of a combination of higher O2 concentration and alkaline pH (8–9) in the chloroplast reaction mixture. We measured the integrity of chloroplasts which had been aerated with 100% O2 or N2 for 12 min in the light with reaction mixture pH at 7.55 or at 8.5. This was done by assaying RuDP carboxylase which had leached out of the disrupted intact chloroplasts during aeration. The same percentage of intact chloroplasts was present after any combination of conditions mentioned above after the 12-min treatment (data not shown), although there was some chloroplast disrup-
The relationship between [H+] concentration and the O₂ inhibition of photosynthesis was also examined as a function of CO₂ concentration. At pH 8.5 as well as at pH 7.8, increased "CO₂" levels diminished the Warburg effect on plastid CO₂ incorporation. However, increased total "CO₂" (CO₂ + HCO₃⁻) included in the more acidic mixtures was much more effective in preventing the O₂ inhibition than when these identical "CO₂" levels were included in the basic mixtures. When the reaction mixture pH was 7.8 (Fig. 2A), the O₂ inhibition of plastid CO₂ assimilation in the presence of 0.47 mm "CO₂" was 63% and this inhibition diminished to 25% in the presence of 5.47 mm "CO₂". In concomitant studies in which the reaction medium pH was 8.5 (Fig. 2B), the O₂ inhibition of plastid CO₂ assimilation was 84% in the presence of 0.47 mm "CO₂" and diminished to only 60% in the presence of 5.47 mm "CO₂". In other experiments not shown, titration of total "CO₂" as high as 10 to 15 mm at pH 8.5 often resulted in complete relief of the O₂ inhibition.

**Effects of pH on Product Formation.** The ¹⁴CO₂ assimilation labeling patterns were examined as a function of O₂ concentration and "CO₂" concentration in relation to the pH of the plastid reaction medium. The labeling patterns in Figure 3 represent time points sampled after 12 min of CO₂ assimilation. All patterns are the result of simultaneous experiments.

With the reaction mixture pH at 7.8 and under anaerobic conditions (Fig. 3A), glycolate represented approximately 9% of the ¹⁴C label with 0.47 mm "CO₂" and diminished only slightly to 6% of the label in the presence of 5.47 mm "CO₂". There was no notable change of isotope labeling in other products as a function of increased "CO₂".

As a result of 100% O₂ aeration (Fig. 3B) at pH 7.8, glycolate represented approximately 30% of the label in the presence of 0.47 mm "CO₂" and diminished to 12% in the presence of 5.47 mm "CO₂". As the glycolate labeling decreased as a function of increased CO₂, increases in ¹⁴C-labeling of most other products were associated with triose-P (glyceraldehyde-3-P and dihydroxyacetone-P) and polyglucan (starch). At pH 7.8, the O₂ inhibitions were, with respect to the presence of 0.47 mm, 2.47 mm, and 5.47 mm "CO₂", respectively, 52%, 34%, and 23% (see Fig. 3 legend).

When the reaction mixture pH was 8.5, and as a result of N₂ aeration (Fig. 3C), glycolate represented 30% of the ¹⁴C label in the presence of 0.47 mm "CO₂" and was diminished to 12% of the label in the presence of 5.47 mm "CO₂". In this system, an increase in total "CO₂" resulted in a 40% increase in polyglucan-labeling as well as a sharp decrease in ¹⁴C-triose-P. As in the preparation in which the pH of the medium was 7.8, polyglucan-labeling was always most pronounced during anaerobiosis (Fig. 3, A and C).

In contrast to the aerobic system at pH 7.8 (Fig. 3B), 100% O₂ aeration of the more alkaline reaction mixture (Fig. 3D) resulted in the incorporation of 58% of the ¹⁴C label into glycolate in the presence of 0.47 mm "CO₂" and the labeling of this intermediate was diminished to 35% of the total with 5.47 mm "CO₂". The decrease in glycolate labeling by increased "CO₂" was associated most predominantly with an increase in the incorporation of label into triose-P (Fig. 3D). At pH 8.5, the O₂ inhibition of chloroplast ¹⁴CO₂ assimilation was, with respect to the presence of 0.47 mm, 2.47 mm, and 5.47 mm "CO₂", respectively, 63%, 61%, and 33%.

Experiments with plastid preparations aerated with 21% O₂ (or N₂) at pH 7.5 compared with pH 8.4 displayed glycolate-labeling trends similar to those studies done with 100% O₂ aeration. After 15 min of ¹⁴CO₂ assimilation in the presence of 21% O₂ and 0.68 mm "CO₂" with pH 7.5, ¹⁴C-glycolate represented 13% of the total ¹⁴C incorporated, and in the presence of N₂ at this pH, ¹⁴C-glycolate contained 6% of the label. In concomitant experiments done at pH 8.5, ¹⁴C-glycolate possessed 26% of the label in the presence of 21% O₂ but had 8% of the label as a result of N₂ aeration. In the experiments with the

**Fig. 1.** O₂ inhibition of chloroplast ¹⁴CO₂ assimilation as a function of pH in the incubation medium. The primary components of the incubation environment were 0.05 M HEPES (pH 7.55 or pH 8.4), 0.33 M sorbitol, 1 mM Na₃P₂O₇, 2 mM EDTA, 1 mM MgCl₂, and 1 mM MnCl₂; the mixture was aerated with streams of 100% O₂ or 100% N₂. The final reaction mixture volumes were 1.05 ml and contained 34.5 µg Chl. The total ³⁴CO₂ was 0.65 mm (40 µCi); with reaction mixture pH at 7.55 the initial concentration of CO₂ (equilibrium at 25 C) was computed to be 25.8 µm, but with pH at 8.4, CO₂ in solution was computed to be 3.8 µm. At pH 7.55, the O₂ inhibition ranged from 54% in the initial 3 min to approximately 22% in the remaining 9 min; with pH at 8.4, the inhibition was approximately 80 to 84% during the entire time course. All kinetic plots are based upon two identical and concomitant determinations for each treatment.

**Fig. 2.** Intact chloroplast ¹⁴CO₂ assimilation as a function of "CO₂." O₂ concentration, and pH in the incubation environment. The primary components of the reaction media are identical to those stated for Figure 1 except as indicated. The exception was that 0.05 M Tricine was employed as buffer to maintain pH at 8.5 (HEPES was used for pH 7.8). The final mixture volumes were 2 ml and contained 108.4 µg of Chl and 80 µCi of ³⁴CO₂. A: With reaction mixture pH at 7.8, the total "CO₂" employed was 0.47 mm (10.7 µM CO₂), 2.47 mm (56.2 µM CO₂), and 5.47 mm (114.3 µM CO₂). O₂ inhibitions computed during 10 to 11 min of light-dependent "CO₂" assimilation as a function of increased "CO₂" levels were 63, 50, and 25%, respectively. B: With reaction mixture pH at 8.5, the total "CO₂" employed was 0.47 mm (2.2 µM CO₂), 2.47 mm (11.4 µM CO₂), and 5.47 mm (25.3 µM CO₂). O₂ inhibitions computed during 10 to 11 min of photosynthesis as a function of increased "CO₂" levels were 84, 66, and 60%, respectively.
"CO₂" level (Fig. 2, A and B and legend of Fig. 3, A and C). Thus the total amount of higher "CO₂" imported into the chloroplast was not limited by the higher pH even though the equilibrium CO₂ levels were computed to be greatly diminished in the more alkaline reaction mixtures (see Fig. 2 legend for values). Poincelot and Day (14) have demonstrated that the chloroplast envelope possesses an active transport mechanism for the rapid accumulation of HCO₃⁻. Also, HCO₃⁻ has been shown to be accumulated in the plastid independent of the pH of the incubation medium, in the pH range 7.5 to 8.5 (13). In conjunction with this, carbonic anhydrate activities in the chloroplast stroma are sufficient to provide CO₂ for RuDP carboxylase regardless of the pH of the external medium or of the stroma (12). Furthermore, J. M. Robinson and R. P. Poincelot (unpublished data) have also shown that carbonic anhydrate isolated from spinach chloroplasts is not inhibited by O₂. Therefore, the increased O₂ inhibition at higher pH must reflect the effect of O₂ upon the plastid carbohydrate metabolism rather than being a reflection of a decrease in available CO₂ in the chloroplast.

The optimal conditions for chloroplast photosynthesis are associated with saturating CO₂ levels, 0 to 21% O₂, and reaction mixture pH in the range 7.5 to 8.5 (Fig. 2 and refs. 5 and 10). Under these conditions where a Warburg effect was not observed, more alkaline as compared with more acidic pH favored the flow of photosynthate to glycolate and away from other Calvin cycle intermediate pools (Fig. 3, A and C, and refs. 4, 10, and 11).

If conditions in the plastid reaction mixture are designed to favor the maximal accumulation of glycolate, then a critical factor is the pH of the reaction environment. Thus, rate-limiting "CO₂" (100% O₂, and a more alkaline pH combined to produce the optimal solution environment for glycolate accumulation during CO₂ assimilation (Fig. 3, B and D)). These are precisely the conditions which favored the most severe O₂ inhibition of CO₂ assimilation (Figs. 2 and 3). A result of the lowered rates of photosynthesis in O₂ at higher pH was the diminutions of substrate pool sizes in the chloroplast. Additionally, the flow of photosynthate from intermediates of the Calvin cycle to provide substrate for glycolate synthesis was strongly enhanced under the more alkaline conditions. The optimal conditions for glycolate synthesis promoted a rapid utilization and lowering of the "CO₂"-phosphorylated intermediate pools, particularly triose-P, and the lack of sugar phosphate appeared to limit starch synthesis (Fig. 3D). The result of this enhanced utilization of phosphorylated intermediates for glycolate synthesis and lowering pH apparently resulted in a strong impediment of RuDP regeneration leading to a more severe inhibition of CO₂ assimilation. Bassham and Kirk (3) have presented evidence that the synthesis of both phosphoglycolate and glycolate diminish the level of RuDP in Chlorella cells. Additionally, evidence from this laboratory (5) as well as from Bassham and Kirk (2) has suggested that a causal factor of the Warburg effect was a block of RuDP regeneration due to the enhanced synthesis of glycolate.

Since the chloroplast actively accumulates HCO₃⁻ regardless of pH (13), it should be reemphasized that increased pH resulted in an optimization of glycolate synthesis, rather than causing a decrease in available CO₂ in the plastid (which would also reflect increased glycolate-labeling). Had higher pH limited the available CO₂ in the plastid, the increased "CO₂" levels at higher pH would have resulted in a much smaller decrease in the CO₂-sensitive glycolate "CO₂"-labeling than was observed for the identical increase in "CO₂" at less basic pH. This was not the case since "CO₂" titration at either pH employed resulted in the same magnitude of decrease in glycolate-labeling (Fig. 3).

The inhibitory effect of increased "CO₂" upon glycolate accumulation was evident at pH 7.8 as well as at pH 8.5 (Fig. 3). There appeared to be both a "CO₂"-sensitive and a "CO₂"-insensitive increment of glycolate synthesis; with O₂ aeration at

**DISCUSSION**

Since pH 8.4 to 8.5, in contrast to pH 7.5 to 7.8, in the chloroplast reaction mixture resulted in an increased severity of the Warburg effect (Figs. 1 and 2), then clearly pH becomes a salient factor associated with O₂ inhibition of photosynthesis. Indeed, using CO₂-dependent O₂ evolution in intact spinach plastids as an assay, Heber et al. (9) observed an increased inhibition by O₂ as a function of increased pH (7.2 raised to 8.3).

The apparent competition of O₂ with CO₂, reflected by the CO₂ assimilation rates in the presence of N₂ or O₂, and as a function of total "CO₂" level, was much stronger when the reaction mixture was more alkaline (Fig. 2). Since the magnitude of the Warburg effect is inversely related to the "CO₂" concentration (5, 7, 19), these data suggest that the effect of higher pH (with constant "CO₂") was associated with a lowering of the available CO₂ for photosynthesis. However, in the absence of O₂, and when the rate of "CO₂" assimilation was examined as a function of increased "CO₂," the rates at pH 7.8, when compared with those at pH 8.5, were nearly equivalent for each
pH 8.5 (Fig. 3D), an increase of "CO₂" from 0.47 mm to 2.47 mm resulted in an approximate 36% decrease in glycolate labeling. In this case, the "CO₂"-sensitive mechanism for glycolate synthesis was almost entirely inhibited by 2.47 mm "CO₂," since raising the "CO₂" from 2.47 mm to 5.47 mm resulted in a decrease of glycolate-labeling from only 37 to 35%. It is clear from our data (Fig. 3) that both the "CO₂"-sensitive and the "CO₂"-insensitive glycolate synthetic mechanisms have an alkaline pH optimum, and both mechanisms require O₂.

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