Plant Physiol. (1977) 59, 530-534

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

I. CARBON DIOXIDE PHOTOASSIMILATION AND GLYCOLATE SYNTHESIS[1]

Received for publication September 15, 1976 and in revised form November 3, 1976

J. MICHAEL ROBINSON,[2] MARTIN GIBBS, AND DONALD N. COTLER[3]
Institute for Photobiology of Cells and Organelles, Brandeis University, Waltham, Massachusetts 02154

ABSTRACT

The influence of pH upon the O_2 inhibition of $^{14}CO_2$ photoassimilation (Warburg effect) was examined in intact spinach (*Spinacia oleracea*) chloroplasts. With conditions which favored the Warburg effect, *i.e.* rate-limiting CO_2 and 100% O_2, O_2 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 ^{14}C-labeling of glycolate, and a decrease of isotope in some phosphorylated Calvin cycle intermediates, particularly triose-phosphate. The ^{14}C-labeling of starch was also more inhibited by O_2 at higher pH. The enhanced synthesis of glycolate during $^{14}CO_2$ 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 ^{14}C-labeling profiles have been interpreted in terms of a "CO_2"-sensitive as well as a "CO_2"-insensitive mechanism for glycolate synthesis. Both mechanisms functioned optimally at the higher pH and both responded to O_2.

Many hypotheses have been proposed to explain the Warburg effect or the inhibition of CO_2 photoassimilation by O_2 (7, 17). One popular explanation is based upon an enhanced synthesis of glycolate in response to O_2 resulting in a diminution of ribulose-1,5-diP[4] generation (2, 3, 5). The consequence would be a decreased CO_2 uptake. Hitherto, attention has been addressed in both whole plants and isolated chloroplasts to glycolate yield and RuDP generation in terms of the concentration of CO_2 and O_2. It has been well established that glycolate levels are directly related to O_2 but inversely to CO_2 (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 CO_2 in air, pH 8.5 in contrast to 7.5 increased 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 CO_2 and O_2. The purpose of the present investigation is not only to evaluate the effect of pH on glycolate yield when the CO_2 and O_2 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 M sorbitol, 1 mM $Na_4P_2O_7$, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$.

Plastid $^{14}CO_2$ Assimilation. Light-dependent $^{14}CO_2$ 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 M sorbitol, 1 mM $Na_4P_2O_7$, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The mixtures were brought to the appropriate pH with 12 N NaOH. The mixtures contained 30 to 50 μg chloroplast Chl/ml. The effects of O_2 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 mM to 5.47 mM total "CO_2" and 40 μCi ^{14}C/ml. The concentration of dissolved CO_2 in the reaction mixtures was computed as a function of pH at 25 C employing the rate constants for CO_2 hydration and HCO_3^- dehydration reported by Gibbons and Edsall (6).

The reaction mixtures were aerated before and during the plastid photosynthesis period with 100% O_2 (approximately 1 mM in solution), 21% O_2 (approximately 0.25 mM O_2 in solution), or 100% N_2. 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 $^{14}CO_2$ assimilation was assayed in a system

[1] This research was generously supported by National Science Foundation Grant BMS71-00978 and United States Energy Research and Development Administration Grant ET(11-1)3231.

[2] Postdoctoral trainee of National Institute of Health Grant BM-1586-09.

[3] Present address: Immunological Research Laboratories, Harvard Medical School, Robert Brigham Hospital, Roxbury, Mass.

[4] Abbreviations: CO_2: CO_2 dissolved in solution; HCO_3^-: bicarbonate; total "CO_2" or "CO_2": CO_2 + HCO_3; RuDP: ribulose-1,5-diP.

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/m^2. 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 ^{14}C-labeled intermediates were measured with gas flow-end window detection as previously described (15). Methods for computation of $^{14}CO_2$ assimilation kinetic data employed have been reported previously (8).

Products of Photosynthesis. Acid-stable, ^{14}C-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_3 solvent (15); autoradiography of chromatograms and other techniques used for product identification were similar to those previously reported from this laboratory (15). Except for ^{14}C-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 or 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 ^{14}C-labeled products. All product fractions represented the total carbon incorporated in an aliquot of reaction mixture after 10 to 12 min of photosynthesis.

The isolation of ^{14}C-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 H_2O, and finally brought to pH 7 with 12 N NaOH. This preparation was passed across a Dowex AG 1-X8 acetate (100–200 mesh) column (0.8 × 7 cm resin bed) at the flow rate of 1 ml/min to allow complete adsorption of ^{14}C products. The column was then washed with 50 ml of deionized distilled H_2O. 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 2 ml were collected and discarded. The next 16 ml of 4 N acetic acid contained all of the ^{14}C-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/l dioxane. The total ^{14}C incorporated into all acid-stable products was determined for the aliquot of $^{14}CO_2$ assimilate fractionated for ^{14}C-glycolate, and the ^{14}C-glycolate to ^{14}C total photosynthate ratio was computed.

Known amounts of ^{14}C-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 ^{14}C-glycolate from these mixtures was 95 to 100%, and recovery controls were carried out with each experiment.

In order to establish that only ^{14}C-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-H_2O-diethylamine 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 ^{14}C-glycerate, unknowns were co-chromatographed with known amounts of standard ^{14}C-glycerate as well as ^{14}C-glycolate. X-ray film autoradiography was employed to identify the labeled products. Regardless of the conditions present in the chloroplast reaction mixture, the 4 N acetic acid eluates were found to contain only ^{14}C-glycolate.

Chloroplast Integrity and Reaction Mixture Aeration. To estimate the effect of O_2 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 O_2 or N_2 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 "CO_2" excluded from these plastid mixtures, they were illuminated as well as aerated for 15 min with streams of 100% O_2 or N_2. At the end of this period, illumination and aeration were terminated, and 5.7 μmol (40 μCi) of "$^{14}CO_2$", 20 μmol $MgCl_2$, 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 O_2 or N_2, 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 O_2 and pH.

RESULTS

Influence of pH upon O_2 Inhibition of CO_2 Assimilation. When the total "CO_2" (0.68 mM) in the reaction mixture was rate-limiting, and the reaction mixtures were treated with a stream of 100% O_2, O_2 in contrast to N_2 inhibited CO_2 fixation 22.1% at pH 7.55 and 84.2% at pH 8.4 (Fig. 1). When the reaction medium was aerated with 21% O_2 (or N_2), photosynthesis was inhibited approximately 14 to 30% at pH 7.5 and 73% at pH 8.4 (data not shown). When the total "CO_2" in solution was 0.68 mM, the CO_2 present at equilibrium was computed to be 27 μM at pH 7.55 and 3.9 μM at pH 8.4.

A feature of the O_2 inhibition of photosynthesis is its reversibility associated with the lowering of the O_2 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 CO_2 was rate-limiting), the replacement by N_2 aeration after 6 min of O_2 aeration during the photosynthesis reaction period (about 46% O_2 inhibition) resulted in immediate and complete relief of the O_2 inhibition. With the reaction mixture pH at 8.5, and after 6 min of aeration with 100% O_2 during the initial photosynthesis period, the O_2 inhibition of plastid photosynthesis (78%) could only be partially relieved (65%) even after 12 additional min of N_2 aeration.

In reference to the limited reversibility of the O_2 inhibition at alkaline pH intact chloroplasts are not disrupted as a result of a combination of higher O_2 concentration and alkaline pH (8–9) in the chloroplast reaction mixture. We measured the integrity of chloroplasts which had been aerated with 100% O_2 or N_2 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-

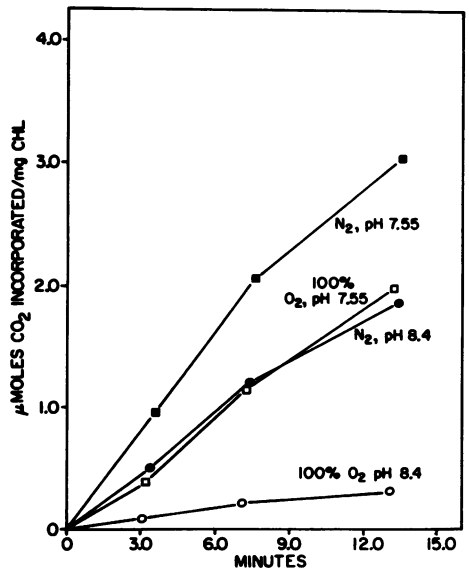

FIG. 1. O_2 inhibition of chloroplast $^{14}CO_2$ assimilation as a function of pH in the incubation medium. The primary components of the incubation environment were 0.05 M HEPES (pH 7.55 or pH 8.4), 0.33 M sorbitol, 1 mM $Na_4P_2O_7$, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$; the mixture was aerated with streams of 100% O_2 or 100% N_2. The final reaction mixture volumes were 1.05 ml and contained 34.5 μg Chl. The total "$^{14}CO_2$" concentration was 0.65 mM (40 μCi); with reaction mixture pH at 7.55 the initial concentration of CO_2 (equilibrium at 25 C) was computed to be 25.8 μM, but with pH at 8.4, CO_2 in solution was computed to be 3.8 μM. At pH 7.55, the O_2 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.

tion due to aeration by N_2 or O_2.

The relationship between $[H^+]$ concentration and the O_2 inhibition of photosynthesis was also examined as a function of CO_2 concentration. At pH 8.5 as well as at pH 7.8, increased "CO_2" levels diminished the Warburg effect on plastid CO_2 incorporation. However, increased total "CO_2" ($CO_2 + HCO_3^-$) included in the more acidic mixtures was much more effective in preventing the O_2 inhibition than when these identical "CO_2" levels were included in the basic mixtures. When the reaction mixture pH was 7.8 (Fig. 2A), the O_2 inhibition of plastid CO_2 assimilation in the presence of 0.47 mM "CO_2" was 63% and this inhibition diminished to 25% in the presence of 5.47 mM "CO_2." In concomitant studies in which the reaction medium pH was 8.5 (Fig. 2B), the O_2 inhibition of plastid CO_2 assimilation was 84% in the presence of 0.47 mM "CO_2" and diminished to only 60% in the presence of 5.47 mM "CO_2." In other experiments not shown, titration of total "CO_2" to as high as 10 to 15 mM at pH 8.5 often resulted in complete relief of the O_2 inhibition.

Effects of pH on Product Formation. The $^{14}CO_2$ assimilation labeling patterns were examined as a function of O_2 concentration and "CO_2" 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 CO_2 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 ^{14}C label with 0.47 mM "CO_2" and diminished only slightly to 6% of the label in the presence of 5.47 mM "CO_2." There was no notable change of isotope labeling in other products as a function of increased "CO_2."

As a result of 100% O_2 aeration (Fig. 3B) at pH 7.8, glycolate represented approximately 30% of the label in the presence of 0.47 mM "CO_2" and diminished to 12% in the presence of 5.47 mM "CO_2." As the glycolate labeling decreased as a function of increased CO_2, increases in ^{14}C-labeling of most other products were associated with triose-P (glyceraldehyde-3-P and dihydroxyacetone-P) and polyglucan (starch). At pH 7.8, the O_2 inhibitions were, with respect to the presence of 0.47 mM, 2.47 mM, and 5.47 mM "CO_2," respectively, 52%, 34%, and 23% (see Fig. 3 legend).

When the reaction mixture pH was 8.5, and as a result of N_2 aeration (Fig. 3C), glycolate represented 30% of the ^{14}C label in the presence of 0.47 mM "CO_2" and was diminished to 12% of the label in the presence of 5.47 mM "CO_2." In this system, an increase in total "CO_2" resulted in a 40% increase in polyglucan-labeling as well as a sharp decrease in ^{14}C-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% O_2 aeration of the more alkaline reaction mixture (Fig. 3D) resulted in the incorporation of 58% of the ^{14}C label into glycolate in the presence of 0.47 mM "CO_2" and the labeling of this intermediate was diminished to 35% of the total with 5.47 mM "CO_2." The decrease in glycolate labeling by increased "CO_2" was associated most predominantly with an increase in the incorporation of label into triose-P (Fig. 3D). At pH 8.5, the O_2 inhibition of chloroplast $^{14}CO_2$ assimilation was, with respect to the presence of 0.47 mM, 2.47 mM, and 5.47 mM "CO_2," respectively, 63%, 61%, and 33%.

Experiments with plastid preparations aerated with 21% O_2 (or N_2) at pH 7.5 compared with pH 8.4 displayed glycolate-labeling trends similar to those studies done with 100% O_2 aeration. After 15 min of $^{14}CO_2$ assimilation in the presence of 21% O_2 and 0.68 mM "CO_2" with pH at 7.5, ^{14}C-glycolate represented 13% of the total ^{14}C incorporated, and in the presence of N_2 at this pH, ^{14}C-glycolate contained 6% of the label. In concomitant experiments done at pH 8.5, ^{14}C-glycolate possessed 26% of the label in the presence of 21% O_2, but had 8% of the label as a result of N_2 aeration. In the experiments with the

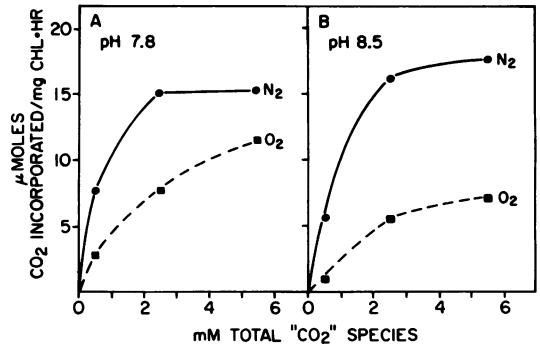

FIG. 2. Intact chloroplast $^{14}CO_2$ assimilation as a function of "CO_2," O_2 concentration, and pH in the incubation environment. The primary components of the reaction media are identical to those stated for Figure 1 except as indicated. The exception was that 0.05 M Tricine was employed as buffer 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 "$^{14}CO_2$." A: With reaction mixture pH at 7.8, the total "CO_2" employed was 0.47 mM (10.7 μM CO_2), 2.47 mM (56.2 μM CO_2), and 5.47 mM (124.3 μM CO_2). O_2 inhibitions computed during 10 to 11 min of light-dependent $^{14}CO_2$ assimilation as a function of increased "CO_2" levels were 63, 50, and 25%, respectively. B: With reaction mixture pH at 8.5, the total "CO_2" employed was 0.47 mM (2.2 μM CO_2), 2.47 mM (11.4 μM CO_2), and 5.47 mM (25.3 μM CO_2). O_2 inhibitions computed during 10 to 11 min of photosynthesis as a function of increased "CO_2" levels were 84, 66, and 60%, respectively.

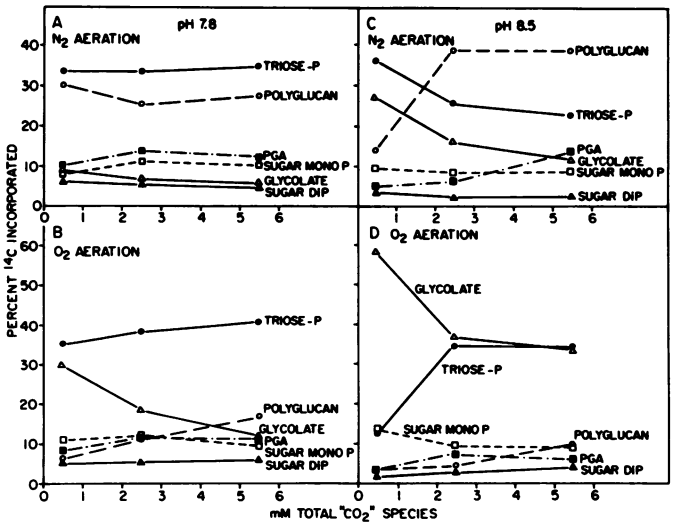

FIG. 3. Photosynthetic ^{14}C-product-labeling patterns associated with 10 to 12 min of intact chloroplast $^{14}CO_2$ assimilation as a function of "CO_2," O_2 concentration, and pH in the incubation environment. The primary components of the reaction media are identical to those stated for Figure 1 except that 0.05 M Tricine was employed as buffer to maintain pH at 8.5 (HEPES was used for pH 7.8). The final reaction mixture volumes were 1 ml and contained 51.4 μg Chl as well as 40 μCi of "$^{14}CO_2$." Total "CO_2" and CO_2 concentrations were identical to those identified in Figure 2. Carbon 14-labeled products of photosynthesis were identified employing paper chromatography and Dowex-acetate chromatography for glycolate purification (see under "Materials and Methods"). Triose-P: glyceraldehyde-3-P and dihydroxyacetone-P; polyglucan: starch (1 N HCl hydrolyzate yields ^{14}C-glucose); sugar mono-P: glucose-1-P, glucose-6-P, fructose-6-P, and ribose-5-P; sugar diP: fructose-1,6-diP and ribulose-1,5-diP. A: With reaction mixture pH at 7.8, and with 100% N_2 aeration, the velocities of $^{14}CO_2$ assimilation were, with respect to increased "CO_2," 4.6, 12.4, and 15.1 μmol CO_2 assimilated/mg Chl·hr. B: With pH at 7.8, and with 100% O_2 aeration the rates of $^{14}CO_2$ assimilation were, with respect to increased CO_2, 2.2, 8.1, and 11.7. The computed severities (10 min) of the Warburg effect were, for increased CO_2, 52, 34, and 23%, respectively. C: With pH at 8.5, and with N_2 aeration, the rates of $^{14}CO_2$ assimilation were, with respect to increased "CO_2," 3.4, 12.7, and 12.9. D: With pH at 8.5, and with O_2 aeration, the rates of $^{14}CO_2$ assimilation were, with respect to increased "CO_2," 1.3, 4.9, and 8.7. The computed severities of the Warburg effect were, for increased CO_2, 63, 61, and 33%, respectively.

reaction mixtures at pH 7.5 and with 21% O_2 (or N_2), inhibition by O_2 was estimated to be 10 to 50%, and at pH 8.4, inhibition was approximately 70% (data not illustrated).

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 O_2 inhibition of photosynthesis. Indeed, using CO_2-dependent O_2 evolution in intact spinach plastids as an assay, Heber *et al.* (9) observed an increased inhibition by O_2 as a function of increased pH (7.2 raised to 8.3).

The apparent competition of O_2 with CO_2, reflected by the CO_2 assimilation rates in the presence of N_2 or O_2, and as a function of total "CO_2" 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 "CO_2" concentration (5, 7, 19), these data suggest that the effect of higher pH (with constant "CO_2") was associated with a lowering of the available CO_2 for photosynthesis. However, in the absence of O_2, and when the rate of $^{14}CO_2$ assimilation was examined as a function of increased "CO_2," the rates at pH 7.8, when compared with those at pH 8.5, were nearly equivalent for each "CO_2" level (Fig. 2, A and B and legend of Fig. 3, A and C). Thus the amount of total "CO_2" imported into the chloroplast was not limited by the higher pH even though the equilibrium CO_2 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 HCO_3^-. Also, HCO_3^- 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 anhydrase activities in the chloroplast stroma are sufficient to provide CO_2 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 anhydrase isolated from spinach chloroplasts is not inhibited by O_2. Therefore, the increased O_2 inhibition at higher pH must reflect the effect of O_2 upon the plastid carbohydrate metabolism rather than being a reflection of a decrease in available CO_2 in the chloroplast.

The optimal conditions for chloroplast photosynthesis are associated with saturating CO_2 levels, 0 to 21% O_2, 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 "CO_2," 100% O_2, and a more alkaline pH combined to produce the optimal solution environment for glycolate accumulation during CO_2 assimilation (Fig. 3, B and D). These are precisely the conditions which favored the most severe O_2 inhibition of CO_2 assimilation (Figs. 2 and 3). A result of the lowered rates of photosynthesis in O_2 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 ^{14}C-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 at higher pH apparently resulted in a stronger impedance of RuDP regeneration leading to a more severe inhibition of CO_2 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 HCO_3^- 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 CO_2 in the plastid (which would also reflect increased glycolate-labeling). Had higher pH limited the available CO_2 in the plastid, then the increased "CO_2" levels at higher pH would have resulted in a much smaller decrease in the CO_2-sensitive glycolate ^{14}C-labeling than was observed for the identical increase in "CO_2" at less basic pH. This was not the case since "CO_2" titration at either pH employed resulted in the same magnitude of decrease in glycolate-labeling (Fig. 3).

The inhibitory effect of increased "CO_2" upon glycolate accumulation was evident at pH 7.8 as well as at pH 8.5 (Fig. 3). There appeared to be both a "CO_2"-sensitive and a "CO_2"-insensitive increment of glycolate synthesis; with O_2 aeration at

pH 8.5 (Fig. 3D), an increase of "CO_2" from 0.47 mM to 2.47 mM resulted in an approximate 36% decrease in glycolate-labeling. In this case, the "CO_2"-sensitive mechanism for glycolate synthesis was almost entirely inhibited by 2.47 mM "CO_2," since raising the "CO_2" 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 "CO_2"-sensitive and the "CO_2"-insensitive glycolate synthetic mechanisms have an alkaline pH optimum, and both mechanisms require O_2.

Acknowledgments – The authors thank T. Bamberger, J. Zweier, and S. Atchley for valuable technical assistance. We thank C. Robinson for excellent clerical and editorial assistance during the preparation of this manuscript.

LITERATURE CITED

1. Bahr, JT, RG Jensen 1974 Ribulose diphosphate carboxylase from freshly ruptured spinach chloroplasts having an *in vivo* K*m* (CO_2). Plant Physiol 53: 39-44
2. Bassham JA, M Kirk 1962 The effect of oxygen on the reduction of CO_2 to glycolic acid and other products during photosynthesis by *Chlorella*. Biochem Biophys Res Commun 9: 376-380
3. Bassham JA, M Kirk 1973 Sequence of formation of phosphoglycolate and glycolate in photosynthesizing *Chlorella pyrenoidosa*. Plant Physiol 52: 407-411
4. Dodd WA, RGS Bidwell 1971 The effect of pH on the products of photosynthesis in $^{14}CO_2$ by chloroplast preparations from *Acetabularia mediterranea*. Plant Physiol 47: 779-783
5. Ellyard PW, M Gibbs 1969 Inhibition of photosynthesis by oxygen in isolated spinach chloroplasts. Plant Physiol 44: 1115-1121
6. Gibbons BH, JT Edsall 1963 Rate of hydration of carbon dioxide and dehydration of carbonic acid at 25 C. J Biol Chem 238: 3502-3507
7. Gibbs M 1969 Photorespiration, Warburg effect, and glycolate. Ann NY Acad Sci 168: 356-368
8. Gibbs M, JM Robinson 1974 Photosynthetic CO_2 incorporation in isolated spinach chloroplasts. *In* A San Pietro, ed, Experimental Plant Physiology. CV Mosby, St Louis pp 13-20
9. Heber U, TJ Andrews, NK Boardman 1976 Effects of pH and oxygen on photosynthetic reactions of intact chloroplasts. Plant Physiol 57: 277-283
10. Krapf G, G Jacobi 1975 The pH dependence of CO_2 fixation and carbon assimilation in isolated spinach chloroplasts. Plant Sci Lett 5: 67-71
11. Orth GM, NE Tolbert, E Jimenez 1966 Rate of glycolate formation at high pH. Plant Physiol 41: 143-147
12. Poincelot RP 1972 Intracellular distribution of carbonic anhydrase in spinach leaves. Biochim Biophys Acta 258: 637-642
13. Poincelot RP 1974 Uptake of bicarbonate ion in darkness by isolated chloroplast envelope membranes and intact chloroplasts of spinach. Plant Physiol 54: 520-526
14. Poincelot RP, PR Day 1976 Isolation and bicarbonate transport of chloroplast envelope membranes from species of differing net photosynthetic efficiency. Plant Physiol 57: 334-338
15. Robinson JM, M Gibbs 1974 Photosynthetic intermediates, the Warburg effect, and glycolate synthesis in isolated spinach chloroplasts. Plant Physiol 53: 790-797
16. Robinson JM, M Gibbs 1975 Effects of pH upon the photosynthesis of glycolic acid and the Warburg effect in intact spinach chloroplasts. Plant Physiol 56: S-140
17. Turner JS, EG Brittain 1962 Oxygen as a factor in photosynthesis. Biol Rev 37: 130-170
18. Zelitch I 1965 The relation of glycolic acid synthesis to the primary photosynthetic carboxylation reaction in leaves. J Biol Chem 240: 1869-1876
19. Zelitch I 1971 Photosynthesis, Photorespiration, and Plant Productivity. Academic Press, New York