Photosynthetic Intermediates, The Warburg Effect, and Glycolate Synthesis in Isolated Spinach Chloroplasts[1]

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ABSTRACT

Increasing levels of CO_2 have been shown to stimulate the rate of photosynthesis, eliminate the oxygen inhibition of photosynthesis (Warburg effect), and decrease glycolate formation in isolated spinach chloroplasts. Ribose 5-phosphate and fructose 1,6-diphosphate at concentrations of 5 to 10 μM also stimulate the rate of plastid photosynthesis and eliminate the Warburg effect. In contrast to the effect of high CO_2 levels, these sugar phosphates have little effect on glycolate formation. Evidence is presented to show that the level of intermediates of the photosynthetic carbon reduction cycle may influence the Warburg effect *in vivo*. It is postulated that the formation of glycolate is not the causal factor of the Warburg effect.

The oxygen-mediated inhibition of photosynthetic CO_2 assimilation and O_2 evolution observed in many intact photosynthetic tissues has been termed the Warburg effect (30, 35). Evidence from several laboratories suggests that, in many photosynthetic organisms, there exists a causal relationship between the inhibition of photosynthesis by O_2 and the "apparent" concomitant increase in glycolate formation at the expense of other photosynthetic products (14–16, 35).

The research in this laboratory has been directed to the study of the Warburg effect and glycolate synthesis in isolated spinach chloroplasts. That photosynthesis in isolated chloroplasts responds to the partial pressures of O_2 was demonstrated by Ellyard and Gibbs (15). The evidence presented indicated that 5 to 15 mM levels of bicarbonate, as well as the addition of 1 mM ribose-5-P and fructose-1,6-diP, could eliminate the Warburg effect. In addition, it was shown that photosynthetic glycolate formation was inversely related to the bicarbonate concentration. The increase in CO_2 concentration resulted in the conservation of carbon incorporated into other cycle intermediates, since the incorporated ^{14}C was found to shift from the glycolate pool to most of the other carbon cycle intermediates, as well as starch.

Subsequently, Plaut and Gibbs (24) demonstrated that 1 mM fructose-1,6-diP and glycerate-3-P diminished the appearance of radioactivity in glycolate during chloroplast assimilation of bicarbonate-^{14}C, but the manner in which these intermediates brought about the apparent inhibition of glycolate formation

remained unclear. The present work clarifies the roles of certain carbon reduction cycle intermediates in both stimulating isolated chloroplast CO_2 fixation and relieving the Warburg effect. This work also examines the effect of these photosynthetic intermediates on glycolate synthesis in the chloroplast and compares this effect with that manifested by bicarbonate on glycolate synthesis. A preliminary report of this work has appeared (26).

MATERIALS AND METHODS

Plant Material. *Spinacia oleracea* (var. Bloomsdale Long-Standing) was propagated in a controlled environment chamber on an 8-hr day with 2000 ft-c of white light at 25 C and a 16 hr night at 18 C. Leaves were selected at random from 6 to 10 week old plants. Spinach leaves were also obtained from 6- to 10-week-old plants in the fields of the Don Domenico Pizzi farms at Waltham.

Chloroplast Isolation and Composition of Reaction Mixture. Spinach chloroplasts were prepared using the method of Jensen and Bassham (19), except that sodium isoascorbate and sodium nitrate were omitted from solutions A and B. Ten grams of deveined leaf material were diced and added to a chilled semi-micro homogenizer; the tissue was homogenized for 5 sec with 50 to 60 ml of chilled (5 C) solution A containing MES buffer, pH 6.1. The resulting homogenate was filtered through 16 layers of cheesecloth or one layer of Miracloth. The filtrate was centrifuged for 50 sec at 2000g, and the resulting pellet was resuspended in 1.0 to 2.5 ml of chilled (5 C) solution B containing HEPES buffer at pH 6.7. Chl was estimated by the method of Arnon (1), and the chloroplast preparations contained 45 to 100 μg Chl per 0.1 ml. The reaction mixture (final volume 1–1.5 ml) utilized for determining CO_2 incorporation usually contained either 0.33 M sorbitol or 0.33 M mannitol, 20 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM $Na_4P_2O_7$, 50 mM HEPES buffer at pH 7.8, 0.7 to 1.8 μmoles of $NaH^{14}CO_3$ (12–18$\mu c/\mu$mole) and chloroplasts containing about 45 to 100 μg of Chl. The reaction mixtures were aerated before and during the reaction period with either 100% N_2, 21% O_2 (compressed air), or 100% O_2. The gas streams were passed through water and soda lime filters before their entrance into the solutions; the gases were passed into the reaction mixtures through plastic drinking straws.

In order to measure the specific radioactivity of glycolate-^{14}C, it was necessary to alter both the leaf tissue homogenizing and the chloroplast reaction media. Each of three 25- to 35-g lots of diced spinach leaves were blended separately for 5 to 15 sec in a chilled semi-micro homogenizing vessel with 75 ml of homogenizing medium (5 C) containing 0.33 M sorbitol, 20 mM NaCl, 5 mM $Na_4P_2O_7$, and 50 mM HEPES, pH 6.8 (G-R medium). The resulting homogenates were filtered through 16

layers of cheesecloth, the filtrates were centrifuged at 2000g for 60 sec, and the resulting pellets were taken up in a combined total of 100 ml of the G-R medium. The suspension was then centrifuged at 2000g for 90 sec, and the resulting pellets were resuspended in G-R medium and combined into a total of 28 ml. The resulting suspension contained 0.47 to 0.81 mg Chl/ml, and 5.0 ml of this suspension was used in a reaction mixture which had a final volume of 50 ml. The reaction medium was identical in composition to the G-R medium except that the HEPES buffer was pH 7.8, and the reaction mixtures also contained 20 to 210 μmoles of NaH[14]CO$_3$ with a specific radioactivity of 6.0 to 19 μc/μmole. In these experiments, the reaction mixtures were aerated with air through Intramedic polyethylene tubing (PE 190) which was submerged in each reaction vessel.

Bicarbonate-[14]C Assimilation. The kinetics of photosynthetic CO$_2$ incorporation were determined by measuring the time course uptake of NaH[14]CO$_3$ during illumination of chloroplasts incubated under various gas regimes. Aliquots of 100 to 200 μl were taken from the reaction mixtures at various time intervals, and the reactions were terminated by the addition of 10 or 20 μl of 24 N formic acid. For the determination of isotopic incorporation into acid-stable products, 25 or 50 μl of the acidified samples were pipetted onto aluminum planchets fitted with lens paper discs, air-dried, and counted on a Nuclear Chicago gas flow end window detector system. The rate of CO$_2$ incorporation was computed from kinetic data obtained for the 5- and 10-min interval, since that 5-min period was usually the mid-point of the highest linear velocity.

Reaction mixtures with volumes of 1.0 to 1.5 ml were incubated in 55 $\times$ 17 mm test tubes held in an illuminated, constant temperature bath at 25 C. Illumination was provided by four 150-w flood bulbs, and the reaction systems were illuminated with white light from the two opposing sides to provide an average incident energy of 1.2 $\times$ 10^4 μw/cm^2 per side. In the glycolate measurement studies, 50 ml of chloroplast reaction mixtures were incubated in 55-ml "lollipop" type vessels (9 cm in diameter and a light path of 1 cm) held in an illuminated, constant temperature bath at 25 C. The average incident energy was 1.2 $\times$ 10^4 μw/cm^2 per side (approximately 2000 ft-c). The dark control vessels were covered with aluminum foil, and these dark controls were incubated in the constant temperature bath simultaneously with the illuminated samples.

Products of Photosynthesis. Carbon 14-labeled intermediates were separated by one- or two-dimensional descending chromatography on Whatman No. 1 paper. Isotopically labeled samples were subjected to one-dimensional paper chromatography in Wood's GW$_3$ solvent (33) containing n-butyl alcohol-n-propyl alcohol-acetone-30% (w/v) trichloroacetic acid-80% (w/v) formic acid (40:20:25:15:25 v/v), and 0.3 g EDTA/100 ml of solvent. Samples containing ^{14}C-labeled photosynthetic intermediates were also subjected to two-dimensional paper chromatography: (a) solvent for the first dimensional development was 233.2 g of crystalline phenol/100 ml of H$_2$O with 0.1 ml of 0.5 M EDTA added prior to use; (b) solvent for the second dimension was n-butyl alcohol-propionic acid-water (10.5:5:7 v/v). After solvent development, the chromatograms were allowed to dry at 20 C, and the dried chromatograms were sprayed with 1 M NaHCO$_3$ to convert glycolic acid to the less volatile salt form.

Dried chromatograms were exposed to Kodak No-Screen x-ray film in order to identify the ^{14}C-labeled products. Product identification was confirmed by co-chromatography using both ^{14}C-labeled and unlabeled known standards; unlabeled phosphate esters of acids and sugars were positioned on chromatograms employing the methods outlined by Smith (29). Radio-

activity in the labeled products that were separated by paper chromatography was quantitated by excising the ^{14}C-labeled areas from the chromatograms, placing the excised areas in scintillation vials with 15 ml of fluid containing 5 g PPO/1 toluene, and counting them in a Beckman LS-150 or LS-250 scintillation counter.

Glycolate Quantitation. In the experiments involved with the measurement of glycolate specific radioactivity, the 50-ml reaction mixtures were first sampled during various time intervals to determine the CO$_2$ fixation rate. To stop the reactions at the terminal time, the balance (48 ml) of each reaction mixture was acidified to pH 2.5 to 2.7 with approximately 4.0 mmoles of HCl. Subsequently, each of the mixtures was titrated to neutrality with NaOH. Utilizing vacuum filtration, each mixture was passed across a 934 AH Reeve Angel glass fiber filter with quantitative washing in order to facilitate the complete separation of the reaction mixture from the chloroplast material.

Fractionation and purification of glycolate was accomplished employing the method described by Zelitch (34). Certain modifications of that technique were introduced. Each reaction mixture filtrate was passed across a Dowex AG 1 $\times$ 8 acetate (100-200 mesh) column (0.8 $\times$ 14 cm resin bed) at the rate of 1 to 2 ml/min. After the absorption step, the resin was thoroughly washed by passing 75 ml of deionized-distilled water across the column to ensure the complete removal of all sorbitol from the resin bed, since the hexitol was found to interfere in the colorimetric assay of glycolate. After the resin bed water wash, the glycolate fraction was eluted (1 to 2 ml/min) from the column with 4 N acetic acid. The first 6 ml of effluent were discarded, and the subsequent 15 ml contained the total glycolate fraction. Glycolate was measured employing the method of Calkins (13) in which 0.1-ml aliquots of the glycolate fractions were reacted with 2 ml of freshly prepared 0.01% 2,7-naphthalenediol in 36 N H$_2$SO$_4$. These mixtures were heated at 100 C for 20 min to produce the 525 nm absorbing color complex. Just prior to absorbance measurements in a Beckman DU spectrophotometer, the developed solutions were diluted with 1 ml of 2 N H$_2$SO$_4$. This dilution increased the sensitivity of glycolate detection slightly, resulting in a linear absorbance from 3 to 150 nmoles. It was found that 0.1 ml-aliquots of 4 N acetic acid eluates from control Dowex acetate columns (no glycolate absorbed) produced an $A_{525\ nm}$ of 0.003 unit or less when these aliquots were developed with the Calkins reagent and compared against 0.1 ml aliquots of 4 N acetic acid treated in the same manner. Glycolate produced in the illuminated systems was always estimated by comparing these samples against samples from identical systems incubated in the dark. In our plastid preparations glycolate was not detected prior to illumination nor was dark synthesis of glycolate observed. At least 90 to 98% of known amounts of unlabeled glycolate, as well as glycolate-^{14}C, was recovered by Dowex-acetate chromatography from 50-ml reaction mixtures. Ribose-5-P or fructose-1,6-diP in the reaction mixtures did not interfere with the glycolate fractionation or assay. These phosphate esters were absorbed on the column but neither eluted from the column with 4 N acetic acid. Paper chromatography in a n-butyl alcohol-n-propyl alcohol-acetone-30% trichloroacetic acid-80% formic acid solvent (40:20:25:15:25 v/v) confirmed that the effluents contained only glycolate-[14]C. Using paper chromatography, glycerate-^{14}C was not detected in the 4 N acetic acid eluate.

EDTA was found to co-chromatograph with glycolate in the 4 N acetic acid fraction. In addition, the chelating agent interfered with the Calkins assays by reacting with the naphthalenediol reagent. Therefore, EDTA was omitted from all homoge-
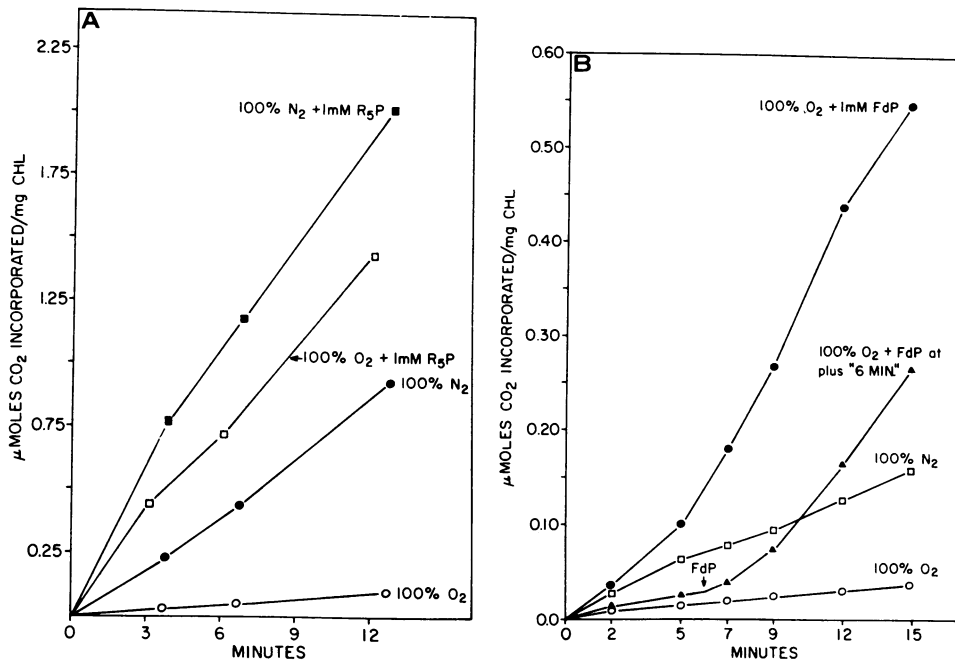
Fig. 1. Effects of ribose-5-P (R5P) or fructose-1,6-diP (FdP) on stimulation of chloroplast CO_2 fixation and relief of the Warburg Effect. The reaction mixtures were aerated with O_2 or N_2. Components of the reaction mixtures are those described in Table I except as follows. A: Ribose-5-P at a final concentration of 1 mM was introduced into the reaction mixtures at 2 min prior to illumination. The final reaction volumes were 1.01 ml and contained 0.75 μmole of bicarbonate and 76.4 μg of Chl. B: Fructose-1,6-diP at a final concentration of 1 mM was introduced into the reaction mixture at 2 min prior to illumination or after 6 min of illumination as indicated. The final reaction volumes were 1.51 ml and these contained 1.8 μmole of bicarbonate and 56.5 μg of Chl.

nizing and reaction mixtures in the experiments involving glycolate assays.

Radioactivity in the glycolate fractions was quantitated by pipetting 25 to 50 μl of the eluate-Dowex fraction onto aluminum planchets fitted with lens paper discs. The planchets were dried at room temperature and were counted in a Nuclear Chicago gas flow end window counter.

RESULTS

Effects of Carbon Reduction Cycle Intermediates on the Stimulation of Plastid Photosynthesis and on the Warburg Effect. Ribose-5-P or fructose-1,6-diP have been shown to stimulate photosynthesis and to reverse the O_2 inhibition of photosynthesis (4, 15, 27). Our results confirmed these previously reported effects and also demonstrated that even in the presence of rate-limiting levels of bicarbonate, 1 mM ribose-5-P as well as 1 mM fructose 1,6 diP will eliminate the O_2 inhibition (Fig. 1A). Additionally, the data presented in Figure 1B demonstrate that 1 mM fructose 1,6-diP can relieve O_2 inhibition rapidly, when the compound is supplied during photosynthesis. These experiments also indicated that gassing of the reaction mixture with O_2 did not destroy plastid integrity, since activity could be restored by the addition of the sugar phosphate during photosynthesis. In addition to the elimination of the Warburg effect, the sugar phosphates produced steady state velocities of photosynthesis in either the presence or absence of O_2 which were approximately 1.5 to 4.0 times greater than those rates observed for plastid photosynthesis in the N_2 control (Fig. 1, A and B). These sugar phosphates apparently have two functions: they protect the plastid's photosynthetic system(s) against O_2 and at the same time increase the rate of photosynthesis.

Previous studies were carried out with millimolar levels of these sugar phosphates. We have observed that the Warburg effect can be eliminated with ribose-5-P (Table I) or fructose-

1,6-diP at concentrations as low as 5 μM (Table II). Concentration requirements were variable and depended upon the environmental regime to which the spinach leaf tissue had been exposed prior to plastid isolation. As shown in Table I, only 5

Table I. *Concentration Requirements of Ribose-5-P for Effects on Plastid Photosynthesis Reflected in Pretreatment of Spinach Leaf Tissue*

Incubation of chloroplasts was carried out in a 1.0-ml reaction mixture which contained 330 μmoles of sorbitol, 50 μmoles of HEPES at pH 7.8, 5 μmoles of $Na_4P_2O_7$, 2 μmoles of EDTA, 20 μmoles of NaCl, 1 μmole of $MgCl_2$, and 1 μmole of $MnCl_2$, 0.74 μmoles of $NaH^{14}CO_3$, 96 μg of Chl (experiment A), and 76 μg of Chl (experiment B) and ribose-5-P. In experiment A, the chloroplasts were isolated from field-grown spinach harvested after exposure to full sunlight for a day. In experiment B, the chloroplasts were isolated from field-grown spinach after placing the leaves in 24 hr of darkness at 7 C followed by 1 hr of light at 2 × 10^3 μw per cm^2. The light intensity was saturating in each experiment and the photosynthetic period was 12 min.

Ribose-5-P	Photosynthetic Rate			
	Experiment A		Experiment B	
	O_2	N_2	O_2	N_2
μM	μmoles CO_2/mg Chl·hr			
0	2.6	3.7	0.6	4.8
5	3.7		1.5	
50	4.4		3.7	
100	4.2		6.1	
500	3.3		7.8	
1000	3.4	4.3	8.8	12.7

μM ribose-5-P was required to relieve completely the rather weak Warburg effect in plastids prepared from spinach leaf tissue immediately taken from high sunlight field conditions, and photosynthesis was also nearly maximal at this concentration. In these plastids, ribose-5-P produced only a slight stimulation of photosynthesis in the presence or absence of O_2. In contrast, photosynthesis in plastids prepared from dark treated ("starved") spinach leaves displayed a rather strong inhibition by O_2 and at least 50 μM ribose-5-P was required to overcome the Warburg effect.

Comparison of Regulation of the Warburg Effect and of the Isotopic Distribution Patterns by Photosynthetic Cycle Intermediates and Bicarbonate. The data in Table II confirm and extend the regulation of the Warburg effect in plastid photosynthesis by the bicarbonate concentration. Thus, 1.3 mM bicarbonate resulted in a 80% inhibition of photosynthesis, while 9.7 mM brought about the complete elimination of this effect. Interestingly, at a bicarbonate level of 1.3 mM that favored the Warburg effect, 5 μM fructose-1,6-diP was required to relieve the O_2 inhibition and produce near maximal stimulation of photosynthesis; on the other hand, only half the amount of fructose-1,6-diP was needed to produce near maximal stimulation of photosynthesis in the presence of N_2 (Table II).

That the relief of the Warburg effect by increasing levels of bicarbonate is accompanied by a decrease in glycolate formation (15-18, 30, 35) has resulted in the notion that a causal relationship exists between glycolate synthesis and the O_2 inhibition of photosynthesis (15, 18, 35). Similarly to bicarbonate, increasing concentrations of fructose-1,6-diP (Fig. 2) or of ribose-5-P (data not shown) eliminate the Warburg effect with a decrease in the percentage of isotopic labeling in glycolate. These findings would indicate that the sugar phosphates and bicarbonate (CO_2) affect the site(s) common to both the O_2 inhibition of photosynthesis and glycolate synthesis.

Coupled to a decrease in isotopic labeling in glycolate as a result of adding ribose-5-P (Table III), fructose-1,6-diP (data not shown), or glycerate-3-P (data not shown) to the reaction mixture was an increase primarily in the labeling in glycerate-3-P and generally in the polyglucan fraction. A similar change

Table II. *Concentration Requirements of Fructose-1,6-diP for Relief of the Warburg Effect and Stimulation of Plastid Photosynthesis as a Function of Bicarbonate Concentration*

Incubation of chloroplasts was carried out in 1.06-ml reaction mixtures which contained 330 μmoles of sorbitol, 50 μmoles of HEPES at pH 7.8, 1 μmole of $Na_4P_2O_7$, 2 μmoles of EDTA, 1 μmole of $MgCl_2$, 1 μmole of $MnCl_2$, 1.3 μmoles of NaH[14]CO3, and 54 μg of Chl (experiment A), 9.7 μmoles of NaH[14]CO3 and 50 μg of Chl (experiment B), and fructose-1,6-diP. The photosynthetic period was 14 min and light intensity was saturating in each experiment.

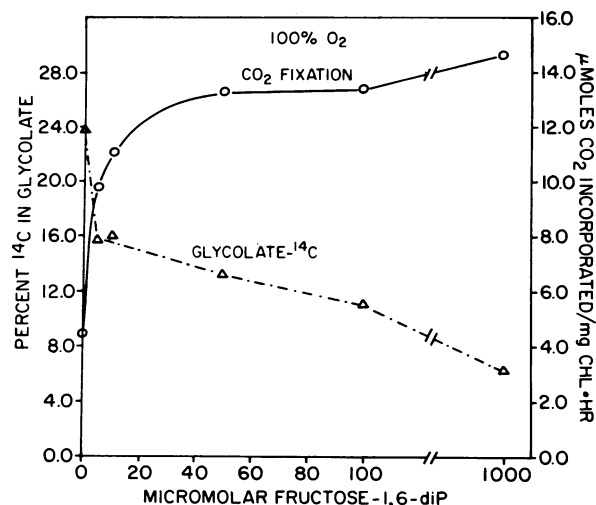| Fructose-1,6-diP	Photosynthetic Rate			
	Experiment A		Experiment B	
	O_2	N_2	O_2	N_2
μM	μmoles CO_2/mg Chl·hr			
0	2.0	9.6	16.3	16.3
2.5	5.7	11.4	25.5	29.0
5	10.3	12.0	30.5	25.1
10	9.4	10.9		
25	8.2	11.7	34.7	29.4
50	14.7	13.7	31.9	26.5
100	11.2	13.8		

FIG. 2. Effect of fructose-1,6-diP concentration upon the O_2 inhibition of CO_2 fixation and on the percentage of ^{14}C incorporated into glycolate. The reaction mixtures of 1 ml were aerated with O_2, and each contained 330 μmoles of mannitol, 50 μmoles of HEPES at pH 7.8, 2 μmoles of EDTA, 0.5 μmole of $MgCl_2$, 0.25 μmole of $MnCl_2$, 0.2 μmole of K_2HPO_4, 1.8 μmoles of NaH[14]CO3, and 52 μg of Chl.

Table III. *Carbon 14 Labeling Pattern in Photosynthetic Products in the Presence of Air as a Function of Ribose-5-P Concentration*

Incubation of chloroplasts was carried out in a 50-ml reaction mixture containing 2.5 mmoles of HEPES, at pH 7.8, 16.5 mmoles of sorbitol, 1 mmole of NaCl, 250 μmoles of $Na_4P_2O_7$, 42.5 μmoles of NaH[14]CO3, 2.33 mg of Chl and ribose-5-P. The sample used for paper chromatography was taken after 35 min of illumination and the light intensity was saturating.

	Ribose-5-P Concn		
	0 μM	50 μM	1000 μM
	% total cpm fixed		
[14]C Intermediates			
Insoluble[1]	8.8	17.3	7.8
Fructose diphosphate	11.1	7.8	5.2
Ribulose diphosphate	10.6	8.3	2.0
Glucose 6-P and fructose 6-P	9.3	9.5	5.5
Ribose-5-P	4.2	5.9	7.2
Glycerate-3-P	11.6	15.8	39.5
Triose phosphate[2]	29.9	27.5	29.1
Glycolate	14.6	8.0	3.8
Rate of fixation, μmoles/mg Chl·hr	2.9	4.7	6.8

[1] Polyglucan.
[2] Mostly dihydroxyacetone-P.

in the ^{14}C-distribution pattern was observed by increasing the bicarbonate concentration (data not shown). Glucose-6-P had no effect on the rate of isotope incorporated during photosynthesis and did not alter the labeling pattern (data not shown).

Finally, we are well aware of the low rates of CO_2 assimilation recorded in the tables. This is primarily due to the elimination of ascorbate and EDTA (previously noted) from the plastid isolation media and reaction mixtures (24). Ascorbate was purposely omitted, since the vitamin alone was found to reverse the O_2 inhibition of photosynthesis (unpublished observation) and furthermore, as did EDTA, to interfere in the Calkins assay for glycolate.

Effect of Sugar Phosphate on the Formation of Glycolate during Photosynthesis. Considerable evidence supports the notion that glycolate is derived from an intermediate of the photosynthetic carbon reduction cycle (2, 5, 7, 8, 10, 11, 14, 28, 32). A number of ketosugars such as fructose-6-P, xylulose-5-P, or sedoheptulose-7-P and the CO_2-acceptor, ribulose-1,5-diP (2, 10, 11, 22, 28, 32) have been proposed as donors of the two-carbon moiety.

Therefore, it would be expected that two-carbon pieces, derived from supplied unlabeled ribose-5-P or fructose-1,6-diP, would be incorporated into glycolate during plastid photosynthesis. Also, if the rate of glycolate synthesis was unaffected or increased by the supplied intermediate (ribose-5-P or fructose-1,6-diP), and unlabeled glycolate molecules were derived from these compounds, then higher levels of these intermediates would cause a decrease in the glycolate specific radioactivity. This would be manifested as a decrease (apparent inhibition) in the percentage of ^{14}C incorporated, especially since glycolate is excreted from isolated chloroplasts (17) and is not further metabolized by the plastid.

Isotopic dilution of the glycolate was tested by measuring the specific radioactivity of the glycolate produced as a function of increasing levels ribose-5-P (Fig. 4) or fructose-1,6-diP (data not shown). For purposes of comparison, we also measured the specific radioactivity of the glycolate as a function of increasing bicarbonate (CO_2) concentration (Fig. 3).

The data in Figure 3 indicate that bicarbonate (CO_2), at levels as low as 4.2 mM, does indeed inhibit glycolate formation in the chloroplast, since both the percentage of ^{14}C incorporated into glycolate, as well as the total glycolate, sharply decrease with increasing bicarbonate (CO_2) concentrations. The resultant ef-
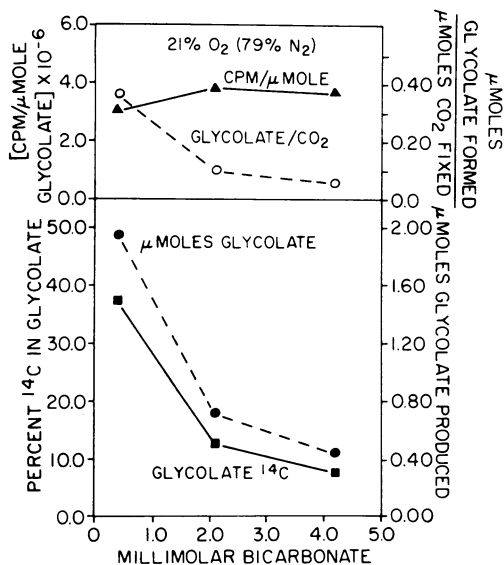

Fig. 3. Effect of bicarbonate concentration on the percentage of ^{14}C incorporated into glycolate and on the total amount and the specific radioactivity of the glycolate synthesized. The reaction mixture was that described in Table III, except that each mixture contained 3.81 mg of Chl in a final volume of 50 ml. Each reaction mixture was aerated with air, the photosynthetic period was 50 min, and the light intensity was 1.2×10^4 μw/cm². The total amount of HCO_3^- incorporated was 5.6 μmoles at 0.42 mM HCO_3^-, 7.6 μmoles at 2.1 mM HCO_3^-, and 7.7 μmoles at 4.2 mM HCO_3^-. The specific radioactivity of the NaH$^{14}CO_3$ initially supplied was, in each case, 6.36 μc/μmole or 2.824×10^6 cpm/μmole (at 20% counting efficiency). The specific radioactivity of glycolate is recorded above as cpm (20% efficiency) per molecule of glycolate (2 carbon atoms per molecule).
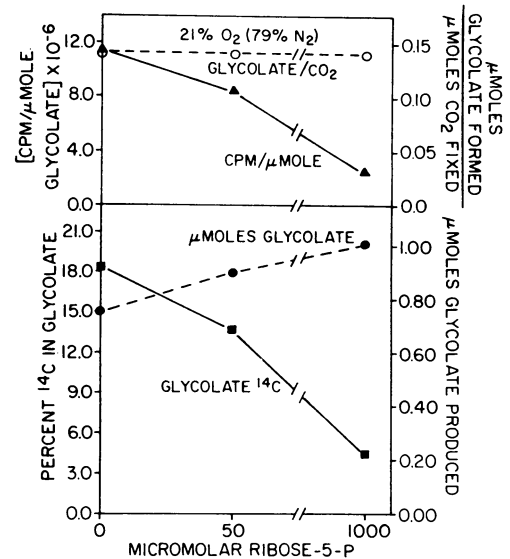

Fig. 4. Effect of ribose-5-P concentration on the percentage of ^{14}C incorporated into glycolate and on the total amount and the specific radioactivity of the glycolate synthesized. The reaction mixture was that described in Table III, except that each reaction mixture contained 2.33 mg of Chl and 42.5 μmoles of NaHCO₃ in a total volume of 50 ml. Each reaction mixture was aerated with air, the photosynthetic period was 35 min, and the light intensity was 1.2×10^4 μw/cm². The total amount of HCO_3^- incorporated was 5.5 μmoles in the control, 6.4 μmoles at 50 μM ribose-5-P, and 7.8 μmoles at 1000 μM ribose-5-P. The specific radioactivity of the NaH$^{14}CO_3$ initially supplied was, in each case, 19.07 μc/μmole or 8.468×10^6 cpm/μmole (at 20% counting efficiency). The specific radioactivity of glycolate is recorded above as cpm (20% efficiency) per molecule of glycolate (2 carbon atoms per molecule).

fect is that the specific radioactivity of the glycolate fraction remained unchanged and not affected by increasing levels of bicarbonate (trace in upper graph of Fig. 3). Since the total CO_2 fixed in the system increased somewhat with increasing bicarbonate levels, the ratio of the total μmoles of glycolate synthesized to the total μmoles of CO_2 incorporated in the same system was sharply decreased as a function of increasing bicarbonate levels. A constant glycolate specific radioactivity indicates that endogenous, and therefore unlabeled, substances within the chloroplast do not yield glycolate under these experimental conditions.

Ribose-5-P or fructose-1,6-diP, in contrast to bicarbonate, show a different effect on glycolate formation. In this case the actual percentage of ^{14}C incorporated into the glycolate fraction was decreased as a function of increasing ribose-5-P (Fig. 4) or fructose-1,6-diP concentrations (data not shown). On the other hand, the μmoles of glycolate synthesized increased with increasing concentrations of ribose-5-P or fructose-1,6-diP. The total CO_2 fixed in the systems also increased to some extent as a function of ribose-5-P (Fig. 4) or fructose 1,6-diP concentration, and thus the ratio of the total μmoles of glycolate synthesized to the total μmoles of CO_2 fixed does not change as a function of increased sugar phosphate (see the traces in upper graph of Fig. 4). The net result is that the specific radioactivity of the glycolate fraction decreased sharply as a function of increasing sugar phosphate concentration.

These data strongly indicate that unlabeled two-carbon pieces, derived and metabolized from the carbon chains of the supplied ribose-5-P and fructose-1,6-diP, are incorporated into glycolate during chloroplast photosynthesis. It is also apparent that higher concentrations of ribose-5-P or fructose-1,6-diP

(50 μM–1 mM), which have been observed to relieve the O_2 inhibition of plastid CO_2 fixation, do not inhibit, but rather increase the formation of photosynthetically derived glycolate.

DISCUSSION

CO_2 Concentration, Photosynthetic Intermediate Concentration, and Relief of the Warburg Effect. That the CO_2 or bicarbonate concentration is of pivotal importance in relief of the Warburg effect in the whole plant is well established (18, 35). Similarly, in the chloroplast, bicarbonate levels less than 1 mM (at pH 7.6–7.8) favor a strong O_2 inhibition of CO_2 fixation (Fig. 1, A and B, and Table II). Although bicarbonate levels in the range of 2 to 10 mM appear to relieve completely this effect on plastid photosynthesis (15 and Table II), the effective concentration varies depending upon the parent tissue.

It is clear from previous work in this laboratory (4, 27), Figure 1, A and B, Tables I and II, and elsewhere (3, 12, 20) that millimolar levels of several photosynthetic intermediates such as glyceraldehyde-3-P, dihydroxyacetone-P, glycerate-3-P, ribose-5-P, and fructose-1,6-diP stimulate the linear rates of CO_2 fixation by intact chloroplasts. It is also clear that these substances remove the initial induction phase lag exhibited by plastid CO_2 fixation by an autocatalytic process associated with filling the photosynthetic intermediate pools (31).

Our studies make it evident that photosynthetic intermediates, such as fructose-1,6-diP, ribose-5-P, or some direct metabolic derivative of these substances, are also effective at micromolar levels in "protecting" against the O_2 inhibition of chloroplast CO_2 fixation (Fig. 1, A and B, Tables I and II). The concentration requirement for "protection" against the Warburg effect was much lower than that concentration requirement for the total stimulation of photosynthesis in 100% O_2 (Table I and II). Levels of supplied intermediates between 5 and 50 μM were effective in totally relieving the Warburg effect at rate-limiting levels of bicarbonate. However, the levels of these supplied intermediates which produced total stimulation often approached 100 to 1000 μM (Table I).

We suggest that the function of increasing CO_2 levels in relief of the Warburg effect may be that of generating the photosynthetic intermediate(s) effective in protection of O_2 sensitive sites. Similarly to bicarbonate, the elimination of the inhibition by a sugar phosphate is rapid (Fig. 1B). Since it is clear from our data that only very low levels (and presumably physiological levels) of exogenously supplied intermediates are required for protection against the Warburg effect (Tables I and II), then it follows that increased CO_2 levels bring about an increased reductive pentose phosphate cycle through increased carboxylation activity. The result would be the synthesis of a sufficient level of the "effective" protective compound in the early seconds of plastid photosynthesis (Fig. 1B). Indeed, if some photosynthetic intermediate or intermediates served this protective function, then the Warburg effect should be rather variable and may be eliminated completely at times of very high photosynthetic activity when the levels of photosynthetic intermediates are high (Table I). It is important to note that while increased levels of CO_2 overcome the Warburg effect, the higher levels of CO_2 do not replace ribose-5-P and fructose-1,6-diP for stimulation of the photosynthetic rate. Sugar phosphates, on the other hand, stimulate steady state plastid CO_2 fixation at both rate-limiting and saturating CO_2 levels (27 and Table II). In reference to our present studies, this point is important because it indicates again that the actual level(s) of photosynthetic intermediates involved in protection of the O_2-sensitive sites appears to be much lower than those associated with the total stimulation of photosynthesis. Sufficient levels of

the effective intermediate for total stimulation (or activation) of photosynthesis are apparently not generated in the presence of saturating CO_2 during the time periods of photosynthesis used in this study. On the other hand, this early period of photosynthesis must be more than sufficient to generate "effective" intermediate levels to eliminate O_2 inhibition at higher CO_2 levels.

Recently, evidence has been presented (9) which would place the site of O_2 inhibition in the Calvin cycle rather than in the photosynthetic electron transport system. Glyceraldehyde-3-P dehydrogenase (15) and ribulose-5-kinase (21) are inhibited by O_2 while there is a report that ribose-5-P affords protection to the enzyme against O_2 inactivation (21). We must now conclude that low concentrations of photosynthetic intermediates are able to provide protection at O_2-sensitive enzyme sites. Preliminary evidence suggests that O_2 actually competes with fructose-1,6-diP, ribose-5-P, or a derived intermediate at the site or sites in the intact chloroplast where this intermediate or a metabolic derivative of this intermediate serves to stimulate light-dependent CO_2 fixation by intact plastids. Approximately 2.5 to 5 μM ribose-5-P or fructose-1,6-diP was found to produce one-half maximal velocity of stimulated plastid CO_2 fixation in the presence of N_2 (at 0.70–1.0 mM HCO_3^- and pH 7.8). Similar values were also obtained in earlier experiments by Harvey and Gibbs (unpublished data). The apparent Km for fructose-1,6-diP stimulation of plastid photosynthesis in the presence of O_2 (at 0.7 mM HCO_3^- and pH 7.8) was 12 μM (data not shown). If oxygen did compete with certain photosynthetic intermediates for sites in the intact chloroplast, then this competition is weak. This would explain why low levels of supplied photosynthetic intermediate were required to "protect" the photosynthetic apparatus of the plastid against O_2 inhibition.

O_2 Concentration, CO_2 Concentration, and Glycolate Synthesis. High rates of light-dependent glycolate synthesis in the chloroplast have a requirement for O_2 (6, 14, 15, 24). However, O_2 concentrations which are favorable to the accumulation of photosynthetically generated glycolate not only inhibit CO_2 fixation (at rate-limiting CO_2 levels), but they also depress the synthesis of almost all Calvin cycle intermediates as well as starch (14, 15). Glycolate synthesis is dependent on the formation of certain photosynthetic intermediates, since glycolate is probably derived from either fructose-6-P, sedoheptulose-7-P, or ribulose-1,5-diP (2, 10, 11, 22, 28, 32). Thus, while O_2 inhibition of photosynthesis appears to favor glycolate accumulation at rate-limiting CO_2 levels, the amount of glycolate accumulation was limited by the rate of Calvin cycle metabolism (Fig. 4).

CO_2 concentration is an extremely critical factor at O_2 concentrations which favor glycolate synthesis. The increase of CO_2 concentration from levels of CO_2 which are rate-limiting for plastid carboxylation to those levels of CO_2 which are saturating, always result in a considerable reduction of glycolate formation (15, 36, Fig. 3), with a concomitant increase in levels of several other Calvin cycle intermediates as well as starch (14, 15). While it is clear that levels of CO_2 which were saturating to photosynthesis reduced the glycolate formation, it is also evident that saturating levels of bicarbonate (CO_2) did not completely eliminate glycolate formation (Fig. 3). This result appears to be inconsistent with the recently proposed oxygenase mechanism for phosphoglycolate formation (2, 10, 22), since this reaction catalyzed by ribulose-1,5-diP carboxylase requires that at least some sites for CO_2 binding must be occupied by O_2. Presumably, all sites for CO_2 binding would be filled with CO_2 molecules at saturating levels of CO_2 and no phosphoglycolate formation should result. Our observations seem more

consistent with the formation of glycolate facilitated by the oxidation of sugar monophosphate via the transketolase mechanism coupled to an oxidant resulting during photosynthetic electron transport (28).

It is important to note that the oxidant involved in photosynthetic glycolate production is thought to be a peroxide, and there is some experimental evidence supporting this contention (28). Furthermore, Patterson and Myers (23) have demonstrated that in *Anacystis nidulans*, photosynthetically linked H_2O_2 production was increased by CO_2 depletion, but higher levels of CO_2 did not completely eliminate H_2O_2 production. We believe that increased saturating CO_2 levels do not eliminate completely the competition for electrons between O_2 and reduced carriers in the spinach plastid. Therefore, increased CO_2 levels may not eliminate glycolate formation completely, even at saturating levels of CO_2.

Photosynthetic Intermediates and Glycolate Synthesis in Intact Chloroplasts. Intact chloroplasts, photosynthesizing in the presence of light and rate-limiting levels of bicarbonate produce a significant glycolate-^{14}C pool which often possesses as much as 50% of the total ^{14}C incorporated depending on the O_2 concentration (15). Since glycolate-^{14}C synthesized during intact chloroplast photosynthesis is derived from photosynthetically produced ^{14}C-labeled sugar phosphate, then unlabeled glycolate also should be derived from supplied ^{12}C-photosynthetic intermediates. Thus, the specific radioactivity of glycolate decreases with increasing levels of exogenous ribose-5-P (Fig. 4), because as the concentration of these intermediates is increased in the presence of plastids incorporating ^{14}CO$_2$, the unlabeled sugar phosphates which are potential precursors of glycolate are in much greater supply than the ^{14}C-labeled sugar phosphate precursors formed as a result of ^{14}CO$_2$ incorporation. In turn, this accounts for the results in Figure 2 which demonstrate that ^{14}C labeling of the glycolate pool decreases as a function of increased fructose-1,6-diP concentration.

Synthesized glycolate is readily excreted from the chloroplast into the reaction mixture, and apparently does not re-enter the organelle (17). It is this export which allows for a reliable measurement of the specific radioactivity of glycolate.

In our experiments, the concentration of supplied intermediate was not rate-limiting to the formation of glycolate. We came to this conclusion because the rate of photosynthesis (Fig. 2), as well as the total carbon fixed (Fig. 4), increased as a function of increasing ribose-5-P or fructose-1,6-diP. We have already noted that these sugar phosphates, or some derivatives of them, are in some way rate-limiting to plastid photosynthesis. It is clear in these studies (Fig. 4) that while the increase of total glycolate appears to be a function of the sugar phosphate concentration, it is, in fact, a function of the over-all photosynthetic activity. Thus, while the total carbon incorporated, as well as the corresponding total glycolate synthesized increased as a function of increasing ribose-5-P or fructose-1,6-diP concentration, the ratio of total glycolate synthesized to total carbon incorporated was not affected by any concentration of supplied sugar phosphate (Fig. 4).

Photosynthetic Intermediate Concentration, Glycolate Synthesis, and the Warburg Effect. Previously, it has been envisaged that the inhibition of photosynthesis by O_2 is coupled to glycolate synthesis (14, 15). This assumption has led some workers to suggest that the ribulose-1,5-diP carboxylase is not only the site of O_2 inhibition, but also the site of P-glycolate (2, 9, 10, 22) and subsequently glycolate formation (25).

Our data casts doubt upon the notion that inhibition of plastid carboxylation is necessarily coupled to glycolate synthesis. It is clear that O_2 inhibition, as well as glycolate synthesis, responds to CO_2 concentration, and levels of CO_2 which are saturating for plastid carboxylation dramatically lower the ratio of glycolate synthesized per total CO_2 incorporated. However, exogenous levels of ribose-5-P or fructose-1,6-diP (5–50 μM) which eliminated totally the O_2 inhibition of plastid carboxylation even at 100% O_2 (Fig. 1, A and B, Tables I and II) did not influence the ratio of glycolate synthesized to total carbon fixed in isolated chloroplasts photosynthesizing under conditions which favor glycolate synthesis (Fig. 4). We must now question the role of glycolate formation as a causal factor of O_2 inhibition, and we must question the necessity for an inhibition of plastid carboxylation in order for glycolate synthesis to proceed. It would seem that there are periods during photosynthesis, especially when certain photosynthetic intermediates are in excess of 100 μM, that the Warburg effect is nonexistent, and at the same time, glycolate formation and presumably photorespiration may proceed unaffected.

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