Kinetic Characteristics of the Chloroplast Envelope Glycolate Transporter†

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ABSTRACT: Studies on the pea chloroplast glycolate transporter have been hampered by the lack of methods capable of measuring initial transport rates. Two novel, multilayered, silicone oil centrifugation techniques have been developed that largely overcome this problem. The distribution of glycolate, at equilibrium, between the chloroplast stroma and the external medium indicates that facilitated diffusion of glycolic acid, symport of a proton and glycolate, and antiport of an hydroxyl ion and glycolate are possible transport mechanisms. The dependence of the kinetic parameters of the transporter on the stromal and medium pH is consistent with mobile carrier models for proton symport (proton binds first) and hydroxyl antiport. A mobile carrier model for proton symport, with random ligand binding, cannot be ruled out. Transport of glycolate by the carrier seems sufficiently rapid to account for in vivo photorespiratory carbon fluxes.

The photorespiratory carbon cycle requires the participation of three membrane-enclosed organelles of the plant cell: the chloroplast, the peroxisome, and the mitochondrion. In the course of this cycle, two molecules of glycolate leave the chloroplast, and one molecule of glycerate is taken up. Although the enzymatic steps of photorespiration have been worked out [see Tolbert (1981) and Ogren (1984) for reviews], relatively little is known about the membrane-transport events that must occur in this metabolic process. Previous reports from this laboratory have presented evidence of the existence of a glycolate transporter in pea chloroplasts (Howitz & McCarty, 1983a,b). It was shown that the rate of glycolate transport, when measured over a sufficiently short time, saturates with increasing glycolate concentration. Pretreatment of chloroplasts with *N*-ethylmaleimide inhibited glycolate transport. This inhibition could be prevented by the presence of glycolate during pretreatment. The structurally similar metabolites glycerate, glyoxylate, and lactate were found to inhibit glycolate uptake. Evidence for a glycerate transporter in spinach chloroplasts has been presented (Robinson, 1982, 1984).

Studies on chloroplast glycolate and glycerate transport have been hampered by the lack of methods capable of yielding good approximations of initial transport rates. In this paper we describe two novel silicone oil centrifugation techniques, which have proven useful in obtaining improved resolution of glycolate efflux and uptake kinetics. We have used these methods to (1) draw inferences about the probable mechanism of the carrier, based on the dependence of its kinetics on stromal and medium pH, and (2) assess the kinetic competence of the glycolate transporter to account for in vivo photorespiratory fluxes.

Experimental Procedures

Plants. Pea plants were grown from seed (*Pisum sativum* var. Progress 9), as described (Howitz & McCarty, 1982). Plants were used for chloroplast preparations 11–14 days after sowing.

Chloroplast Isolation. Intact chloroplasts were isolated by centrifugation through a 40% Percoll layer (Mills & Joy, 1980). The chloroplast pellets (2–3 mg of Chl from 50 g of pea shoots) were resuspended in a total of 20 mL of the following medium: 330 mM sorbitol, 25 mM KCl, 2 mM EDTA,[1] 2 mM $MgCl_2$, 1 mM $MnCl_2$, and 5 mM MOPS–NaOH, pH 7.5. For experiments in which a $HgCl_2$ "inhibitor stop" was to be used, the EDTA and $MnCl_2$ were omitted, and 1 mM $MgCl_2$ was used. The resuspended chloroplasts were centrifuged at 1600*g* for 30 s, and the pellet was resuspended in the same medium to a concentration of about 1 mg of Chl/mL. The intactness (>85%), purity, and internal volume (ca. 27 μL/mg of Chl) assays have been described (Howitz & McCarty, 1982, 1983a). Chlorophyll was measured spectrophotometrically (Arnon, 1949).

Treatment of Chloroplasts prior to Transport Measurements. A wide range of medium and stromal pHs was used. The medium described above was used for all chloroplast resuspensions and for various media containing Percoll (see Silicone Oil Centrifugation Techniques). The buffers added to this basic medium (replacing 5 mM MOPS) and their corresponding pHs (at 4 °C) were as follows: 25 mM citrate–NaOH, pH 5.0 and 5.5; 50 mM MES–NaOH, pH 6.0, 6.5, and 6.9; 50 mM PIPES–NaOH, pH 7.0; 50 mM MOPS–NaOH, pH 7.5; 50 mM HEPES–NaOH, pH 8.0 and 8.3. Pyocyanine (20 μM), an electron-transport mediator, was included in the pH 8.3 medium to help increase the pH of the stroma of illuminated chloroplasts. Unless otherwise indicated, all measurements of glycolate transport, stromal pH, and internal chloroplast volume were performed with illuminated chloroplasts at 4 °C. Seventy-five microliters of the chloroplast suspension (ca. 1 mg of Chl/mL) was added to 675 μL of the appropriate medium, and the tubes were illuminated for 3 min in an ice–water bath. Aliquots of this suspension (100 μL) were transferred to six 400-μL polypropylene microcentrifuge tubes, also on ice and under illumination. The intensity of both illumination was greater than 6×10^5 erg cm^{-2} s^{-1}. The transfers took approximately 3 additional min. Six tubes were

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[1] Abbreviations: MOPS, 4-morpholinepropanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-2-yl)-benzene; [^{14}C]DMO, 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione; NEM, *N*-ethylmaleimide; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Chl, chlorophyll; EDTA, ethylenediaminetetraacetic acid.

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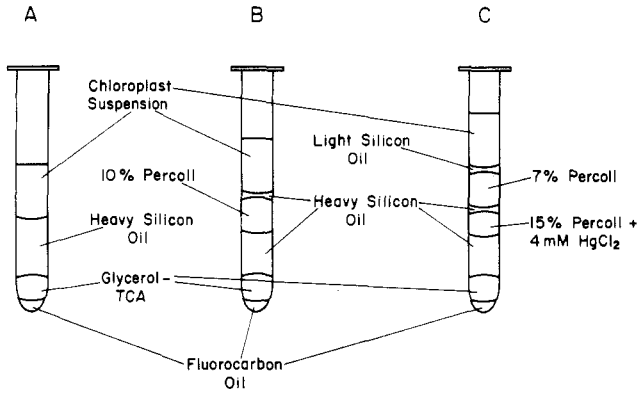

FIGURE 1: Silicone oil centrifugation systems. Volumes and compositions of the various layers are as follows: fluorocarbon oil, 20 μL, density = 1.85 g/mL; glycerol–TCA, 50 μL, 20% (v/v) glycerol, 2% (w/v) trichloroacetic acid; heavy silicone oil, 95 μL (tube A), 70 μL (lower layer, tubes B and C), 25 μL (upper layer), General Electric Versilube F-50 and Dow Corning silicone fluid 702, 8:3 by weight; 10% Percoll layer, 75 μL; 15% Percoll layer, 50 μL; 7% Percoll layer, 75 μL; light silicone oil, 30 μL, Versilube F-50 and General Electric SF 96, 2:1 by weight, chloroplast suspension, 100 μL.

centrifuged together, for 30 s at full speed, in a Beckman microfuge B. All of the operations described above were performed in a cold box (5 °C). The microcentrifuge was modified to allow the installation of a copper cooling coil, which carried a circulating antifreeze mixture at −5 °C. The temperature of the contents of the microcentrifuge tubes, measured with a thermocouple probe (Yellow Springs Instruments Model 42SC), was 4 °C before centrifugation and 5–7 °C after.

Silicone Oil Centrifugation Techniques. Three basic arrangements of aqueous and oil layers in microcentrifuge tubes were used (Figure 1). Sedimented chloroplasts came to rest on top of the fluorocarbon oil layer (a "false bottom" that allowed the tubes to be cut above and below the pellet layer, facilitating resuspension for scintillation counting). The tube type in Figure 1A was used for measurements of chloroplast internal volume, stromal pH, and equilibrium glycolate uptakes. Tubes containing a 10% Percoll layer (Figure 1B) were used primarily for glycolate efflux experiments. A chloroplast suspension that had been incubated with [1-^{14}C]glycolate and [^{3}H]sorbitol was loaded onto three tubes of type A and three of type B. The pellets in the type A tubes would thus contain the initial internal glycolate concentration, and those in type B would contain the concentration remaining after excretion into the 10% Percoll layer. The oil layer separating the chloroplast suspension from the 10% Percoll layer was of the same density as the lower silicone oil layer. Therefore, when centrifugation began, it moved down and merged with the lower oil layer, and the chloroplasts moved freely into the 10% Percoll layer. The tube type depicted in Figure 1C was used for glycolate uptake experiments. [1-^{14}C]Glycolate and [^{3}H]sorbitol were present in the 7% Percoll layer. The 15% Percoll layer, which contained 4 mM $HgCl_2$, served as an inhibitor stop and wash (see Results). The oil layer between the 7% and 15% Percoll layers moved down during centrifugation; the light oil layer, between the chloroplast suspension and the 7% Percoll layer, moved up. The characterization of the performance of these systems is described under Results.

Sample Processing, Radioisotopes, and Scintillation Counting. Where appropriate, supernatants, and samples of Percoll layers were saved for scintillation counting. The upper layers of each tube (all except the fluorocarbon oil, glycerol–trichloroacetic acid, and lowest silicone oil layers) were removed by aspiration. The tubes were frozen on dry ice and cut with a hot knife through the fluorocarbon oil layer and the silicone oil layer. The tube slices containing the pellet and the glycerol–trichloroacetic acid layer were placed in 1.5-mL Eppendorf tubes with 0.95 mL of water. These were later mixed vigorously and centrifuged to sediment the oils and denatured chloroplast membranes.

A sample of the supernatant fluid (0.8 mL) was added to 7 mL of scintillation fluid [33% (v/v) Triton X-100, 67% (v/v) xylenes, 5 g/L PPO, 0.1 g/L POPOP]. Scintillation counting was done on a Beckman Model LS-7500 with the ^{3}H window set at levels 0 and 200 and the ^{14}C window at levels 397 and 655. Curves correlating quenching of ^{3}H counts and ^{14}C counts and spilldown of ^{14}C counts into the ^{3}H channel with *H* number (Compton edge shift) were prepared and used in correction of the raw data.

The internal chloroplast volume was calculated from the difference of the $^{3}H_2O$ space and the [^{14}C]sorbitol or [^{14}C]-sucrose space (Heldt, 1980). For purposes of calculating the stromal glycolate concentration or the stromal pH from [^{14}C]DMO uptake, the stroma was assumed to be 87% of the total chloroplast volume (Heldt, 1980).

In the glycolate transport experiments, the specific activity of [1-^{14}C]glycolate was varied from 5 to 50 mCi/mmol. Net ^{14}C counts per pellet (counts over and above those brought down by trapped medium) did not fall below 100 cpm (ca. 5 times background). Only one specific activity was used within each experiment. The [^{3}H]sorbitol activities were about 6 times those of [1-^{14}C]glycolate. Stocks of sodium [^{12}C]-glycolate were prepared and standardized by titration of glycolic acid with 1.0 N NaOH.

Materials. Percoll and fluorocarbon oil (Fluorinert FC-40) were obtained from Sigma. [1-^{14}C]Glycolate and [U-^{14}C]-sucrose were from both Amersham and ICN. D-[1-^{3}H(N)]-Sorbitol and D-[U-^{14}C]sorbitol were obtained from New England Nuclear.

RESULTS

ΔpH and Glycolate Accumulation. Figure 2A illustrates the relationship between the ΔpH (stromal pH–medium pH), across the chloroplast envelope, determined by the distribution of [^{14}C]DMO and the ratio of stromal to medium glycolate concentration. At equilibrium, glycolate is distributed across the envelope in a way that is very similar to that of DMO. Thus, the internal and external glycolic acid concentrations are equal, and glycolate must be transported either as glycolic acid or via glycolate/H^+ symport or glycolate/OH^- antiport. Consistent with these possible mechanisms, addition of glycolate to a weakly buffered chloroplast suspension causes an increase in the pH of the medium (Howitz & McCarty, 1983b). Like the uptake of [1-^{14}C]glycolate, this pH increase is prevented by a pretreatment of the chloroplast with *N*-ethylmaleimide (NEM).

The data in Figure 2A were obtained with medium glycolate concentrations of 0.1 mM or less. At higher external glycolate concentrations, the glycolate accumulation ratio often fell below the value predicted by the ΔpH (Figure 2B). This occurs in spite of the fact that the measurements of stromal pH by [^{14}C]DMO uptake were done in the presence of [^{12}C]glycolate concentrations equal to those in the corresponding [^{14}C]glycolate uptake measurements. This phenomenon could be caused by a diffusional leak of the glycolate anion. However, when the stromal and medium pHs are equal, both the transporter-mediated movement of glycolic acid equivalents and a leak- (or second carrier) mediated movement of glycolate anion should act to make the glycolate accumulation ratio equal to 1. As can be seen from the data corre-

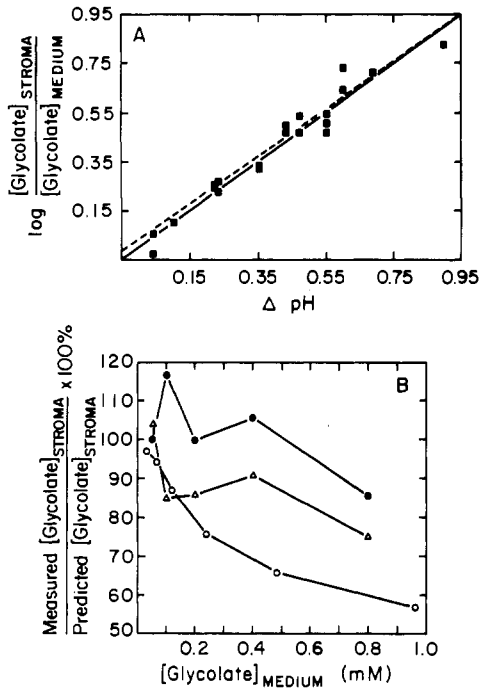

FIGURE 2: (A) Log of the stromal to medium glycolate concentration ratio vs. the ΔpH across the chloroplast envelope (stromal pH–medium pH). Illuminated chloroplasts were incubated with [1-^{14}C]glycolate or [^{14}C]DMO for 6 min before centrifugation. Data were obtained in 11 separate experiments at medium pHs ranging from 6.3 to 8.5. All medium glycolate concentrations were 0.1 mM or less. Solid line is log ($[glycolate]_{stroma}/[glycolate]_{medium}$) = ΔpH. Broken line is the best fit to the data by the method of least squares. (B) Stromal glycolate concentration as a percentage of the concentration predicted by the ΔpH, vs. medium glycolate concentration. Incubation conditions were as in (A). Stroma and medium pHs in three experiments were as follows: (●) stroma 7.47, medium 7.00; (Δ) stroma 7.60, medium 7.60; (○) stroma 8.65, medium 8.30.

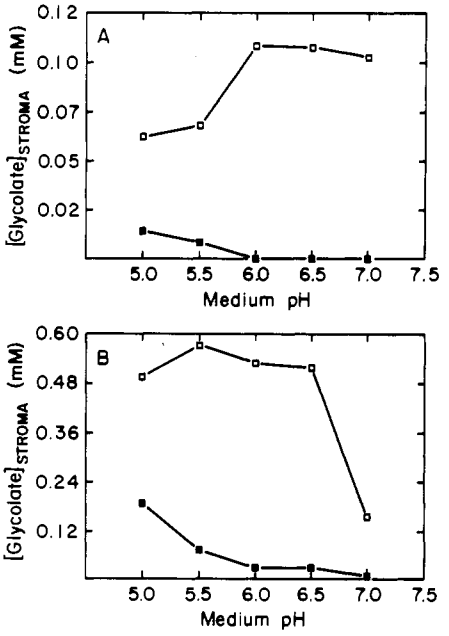

FIGURE 3: Uptake of glycolate by NEM-treated and untreated chloroplasts as a function of pH. NEM treatment (10 mM NEM, 20 min, 2 °C) was done in the resuspension medium described under Experimental Procedures, but which contained 50 mM Tricine/NaOH, pH 7.9, and 1 mg/mL bovine serum albumin. Chlorophyll was 0.15 mg/mL. Treatment was stopped by addition of dithithreitol (10 mM). [1-^{14}C]Glycolate concentration was 0.2 mM in both assays. Both assays were done in the dark. (A) Stromal glycolate concentration after 2 s. Tubes of type B (Figure 1) were used. (□) Untreated chloroplasts; (■) NEM-treated chloroplasts. (B) Stromal glycolate concentration after 75 s. Tubes of type A (Figure 1) were used. (□) Untreated chloroplasts; (■) NEM-treated chloroplasts.

sponding to this case in Figure 2B, this does not seem to be true. As will be shown in a subsequent section, rapid glycolate uptake kinetics appear to be adequately described by a saturable, single K_m, single V_{max} model. A recent discussion of falling accumulation ratios in cotransport systems (Eddy, 1982) has pointed out the advantages of a "slip" model (one in which the carrier–substrate complex can cross the membrane without the proton) in accounting for this phenomenon.

Transport of Glycolic Acid at Low pHs. To test the possibility that glycolic acid can diffuse across the chloroplast envelope without the carrier, NEM-pretreated and untreated chloroplasts were allowed to take up glycolate, for 2 and 75 s, at pHs from 5 to 7 (Figure 3). The experiment was done in darkness, because preliminary unpublished experiments showed that NEM treatment slightly affected the stromal pH of illuminated but not of unilluminated chloroplasts. At 2 s, the NEM-treated chloroplasts did not accumulate glycolate at pH 6 and above. We have shown previously (Howitz & McCarty, 1983a) that a milder NEM treatment (5 mM, 10 min) prevented uptake from pH 7 to pH 8. At 75 s the treated chloroplasts show a pH-dependent uptake over the entire range. If these uptakes are replotted as rates vs. the millimolar concentration of glycolic acid, a linear relationship is obtained (correlation coefficient 0.996) with only a small residual rate (intercept 1.8×10^{-4} mM s^{-1}), which is not dependent on the glycolic acid concentration. The slope of this plot is 0.17 s^{-1} and presumably represents the millimolar rate constant for diffusion of glycolic acid across the chloroplast envelope at 4 °C. All of the kinetic data we will present hereafter was obtained at pHs of 6.5 and above. Given the low concentration of glycolic acid (pK_a = 3.83) under these conditions, the NEM-insensitive glycolic acid diffusion made no significant contribution to the measured transport rates.

The difference in the pH vs. uptake profiles for the untreated chloroplasts at 2 and 75 s reflects the difference between a rate measurement (2 s) and a final extent of uptake measurement (75 s). As will be shown in a later section, lowering the pH of the stroma decreases the V_{max} for uptake. Acidification of the stroma, by incubation at pHs 5.0 and 5.5, would thus explain the lower uptake at 2 s at those pHs. Were the stromal pH constant, the final extent of uptake would be expected to increase exponentially as the medium pH was lowered. The fact that the 75-s uptakes do not increase in this manner is another indication that the stroma has been acidified by incubation at the lower pHs.

Development of Methods for Rapid Chloroplast Transport Measurements. The initial observation of the substrate saturability of glycolate transport rates was made by using 3-s incubations, with unilluminated chloroplasts, at room temperature (Howitz & McCarty, 1983a). The time course of uptake, however, was complete in 10 s or less. Thus, the rates obtained were far from good approximations of initial rates. We attempted to correct this problem by using illuminated chloroplasts (to raise the stromal pH and, therefore, the final extent of uptake) and by working at 4 °C. Glycolate uptake was initiated by injection of a solution, containing the radioisotopes, through a hole bored in the side of the microcentrifuge tube (Howitz & McCarty, 1982). Even with this method, the best results that could be obtained were to observe an uptake 40%–50% of the final extent, at the shortest possible incubation times (2–3 s). The injection method had the additional disadvantages of being poorly reproducible and tedious. Good mixing was not always obtained, and only one tube could be processed at a time.

Table I: Equilibration of Chloroplasts with Percoll Layers during Centrifugation[a]

% Percoll in lower layer	trapped medium from lower layer (%)	% of control vol
0	75	54
5	91	98
10	93	99
15	94	110

[a] Chloroplast suspensions (100 μL, 0.1 mg of chloroplasts/mL) were placed in type B tubes (Figure 1). Suspensions containing [^{3}H]sorbitol were placed in tubes with lower layers containing [^{14}C]sorbitol. The percentage of trapped medium in the pellet derived from the lower layer ([^{14}C]sorbitol space) was calculated. Suspensions containing no radioisotopes were placed in tubes with lower layers containing ^3H_2O and [^{14}C]sorbitol. The ^3H_2O – [^{14}C]sorbitol spaces from these tubes were compared to controls in which the chloroplast suspension was incubated with the same concentrations of ^3H_2O and [^{14}C]sorbitol (percent of control volume). Internal volume of control chloroplasts was 35 μL/mg of Chl.

Initial rate kinetic parameters may also be obtained from the efflux of glycolate from chloroplasts, preloaded in the light, into a medium of very low (ideally zero) glycolate concentration. To do this, microcentrifuge tubes containing a 10% Percoll washing layer (Figure 1, tube B) have been used. The use of different density layers for rapid transport measurements by silicone oil centrifugation was described some years ago (Klingenberg & Pfaff, 1967). The extent to which chloroplasts equilibrate with higher density layers has been assessed in two ways. The first way was to place the labels for the measurement of internal volume not in the chloroplast suspension but in the denser layer through which the chloroplasts would pass. The second approach was to place one marker for trapped medium ([^{3}H]sorbitol) with the chloroplast suspension and a second ([^{14}C]sorbitol) in the denser layer. The results of these tests with 0%–15% Percoll layers are given in Table I. By the criterion of the internal volume measurements, layers of 5%–15% Percoll equilibrate well with the chloroplasts passing through. The discrepancy between this measurement and the measurement of percent trapped medium from the denser layer may in part be due to the longer time the chloroplasts spent in the presence of the [^{3}H]sorbitol (3 min vs. 2 s for [^{14}C]sorbitol). Apparent slow permeation of labeled sorbitol into chloroplasts has been observed (Beck et al., 1983).

Since chloroplasts illuminated in the presence of glycolate achieve an internal concentration approximately 2–5 times that of the medium, the concentration of glycolate in the 10% Percoll layer in an efflux experiment can be no more than a few percent of the initial stromal concentration. For purposes of analyzing our efflux data, we have assumed the glycolate concentration in the 10% Percoll layer to be zero. This assumption allows the Michaelis–Menton equation to be integrated, resulting in the equation (Segel, 1975)

$$S_E - S_t = -K_m \ln (S_E/S_t) + V_{max}t$$

where S_E represents the equilibrium (initial) stromal glycolate concentration and S_t is the the concentration after passage through the 10% Percoll layer, which takes t seconds. Thus, a plot of $S_E - S_t$ vs. ln (S_E/S_t) yields estimates of K_m (–slope) and $V_{max}t$ (intercept). An estimate of the incubation time, t, was obtained by stopping centrifugation at intervals and removing successive 25-μL layers from the top of the supernatant for measurement of their chlorophyll content. Centrifugation was stopped with an electronic timer to turn off the motor and rubber stopper bolted to the lid of the centrifuge as a brake. The rotor could be stopped within 0.2 s after the motor was shut off. The first entry of chloroplasts into the silicone oil

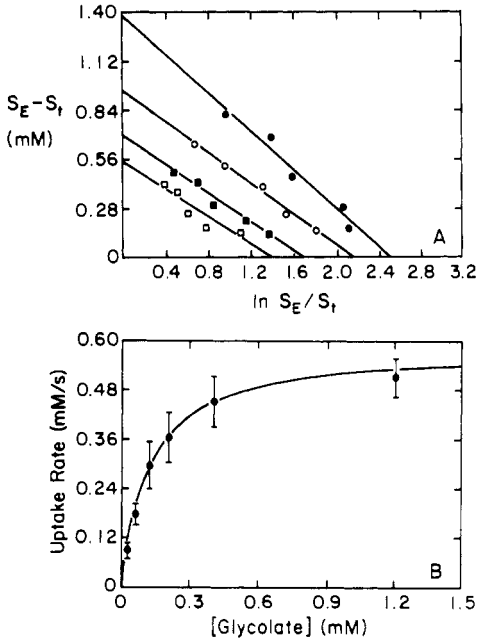

FIGURE 4: (A) Efflux data plots. Stromal pH was 7.5. Medium pHs were 7.1 (□), 7.4 (■), 7.7 (○), and 8.0 (●). Lines are the best fits from the method of least squares. (B) Uptake rates vs. medium glycolate concentration. Stromal pH was 8.6, and medium pH was 7.0. The best fit to the Michaelis–Menton equation was obtained with a nonlinear least-squares program (Marquardt, 1963), on an IBM Personal Computer. The apparent K_m was 0.12 mM, and the apparent V_{max} was 0.59 mM/s [49 μmol (mg of Chl)$^{-1}$ h^{-1}].

occurred in 1.9–2.0 s. From measurement of the chlorophyll content in the layers of the supernatant, it was determined that over 50% of the chlorophyll that entered the 10% Percoll layer had been pelleted 2 s later and over 80% had been pelleted 3 s later. The intactness of our preparations, and hence the pelletable chlorophyll, is 85%–95% (Howitz & McCarty, 1982, 1983a). Since the first pelleting occurred at 2 s, while the rotor is still accelerating, some chloroplasts may pass through the 10% Percoll layer in less than 2 s. Although the chloroplasts do not pass through the 10% Percoll layer in a single, discrete time, calculations based on our data show that a symmetrical distribution of centrifugation times (e.g., 10%, 0.5 s; 20%, 1.25 s; 40%, 2 s; 20%, 2.75 s; 10%, 3.5 s) yields a linear plot with a slope and intercept negligibly different from a plot obtained from a single time that is the mean of that distribution. In estimating V_{max}s from efflux data we have assumed t to be 2 s. Examples of efflux data plots are shown in Figure 4A.

Given the approximations and assumptions inherent in obtaining kinetic parameters from our efflux data, an independent approach to the study of the transporter kinetics was desirable. In the course of obtaining the time estimate for passage of chloroplasts through a 10% Percoll layer, it was noticed that roughly half of this time is spent at the oil–water interface. Thus, if transport could be stopped without the necessity for the chloroplasts to enter an oil layer, the incubation time could be substantially shortened. We reported previously that $HgCl_2$ was a potent inhibitor of glycolate transport (Howitz & McCarty, 1983b). In attempting to use $HgCl_2$ as an inhibitor stop in a conventional way (injection into a stirred suspension of chloroplasts after injection of [^{14}C]glycolate), it was found that most of the glycolate taken up leaked out in the several minutes required to isolate the chloroplasts (K. T. Howitz and R. E. McCarty, unpublished experiments). However, if $HgCl_2$ was included in a denser layer in a microcentrifuge tube, beneath the chloroplast suspension, most of their internal glycolate was retained. This

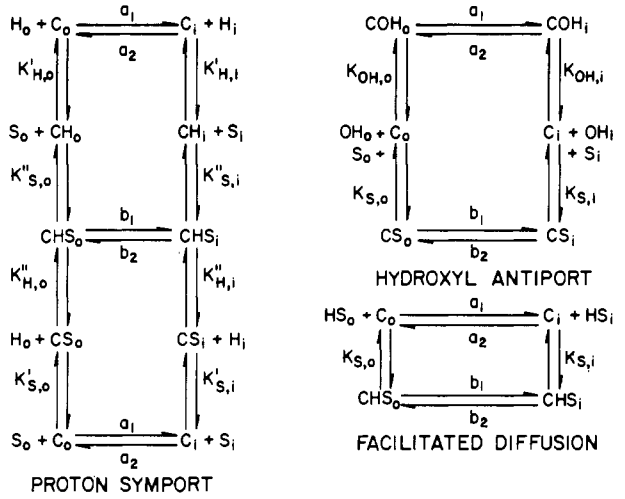

FIGURE 5: Mobile carrier kinetic models for chloroplast glycolate transport. S, H, and OH represent glycolate, protons, and hydroxyl ions on the outside or inside of the chloroplast. C_o and C_i represent the carrier. K's are carrier–ligand dissociation constants for glycolate, protons, hydroxyl ions, or glycolic acid. a_1, b_1, etc. are velocity constants for movement of the carrier (or carrier–substrate complex) across the membrane. Adapted from DeBruijne et al. (1983).

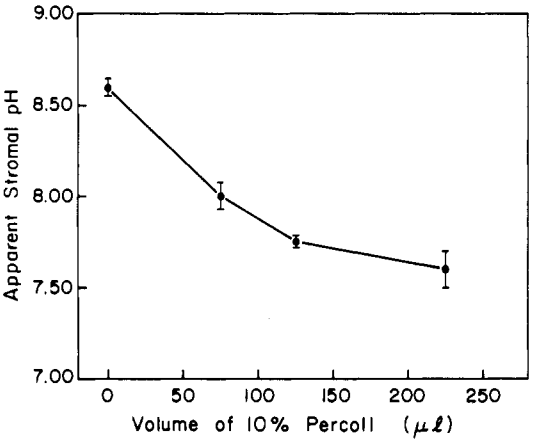

FIGURE 6: Effect of centrifugation through medium of pH 8.0 (10% Percoll) on the stromal pH of chloroplasts preincubated at pH 6.9. See text for experimental conditions and explanation of "apparent stromal pH".

effect was found to saturate around 2 mM $HgCl_2$; 4 mM $HgCl_2$ was used in all experiments presented here. Chloroplasts were equilibrated with [^{14}C]glycolate and then pelleted in either a conventional silicone oil tube (tube A, Figure 1) or a tube (tube C, Figure 1) containing a 7% Percoll layer with the same [^{14}C]glycolate concentration and a 15% Percoll layer with no glycolate and 4 mM $HgCl_2$. The concentration of glycolate in chloroplasts that had passed through the 15% Percoll–$HgCl_2$ layer ranged from 81% to 106% (mean ± SD = 93 ± 6%) of those centrifuged directly into silicone oil. Retention of glycolate was found to be independent of stromal and medium pH and glycolate concentration (not shown). The time for chloroplasts to pass through the 7% Percoll layer was found to be about 1 s.

Although there is some variability in glycolate retention in the inhibitor stop experiments, the method has two distinct advantages. One is the improvement in the estimation of initial uptake rates due to the shorter incubation time. The other is that the washing that chloroplasts receive from the 15% Percoll layer eliminates most of the correction necessary for trapped medium ([^{3}H]sorbitol counts), a source of error in itself. The uptake data are presented without correction for losses incurred by the chloroplasts passing through the 15% Percoll layer. An example of uptake rate data obtained by this method is shown in Figure 4B.

The data that will be presented on the K_ms and V_{max}s for glycolate uptake were derived from 96 different rate determinations (each the mean from three tubes), which were part of 16 separate concentration series experiments. The number of uptakes out of that 96 which represent various percentages of the final extent of glycolate accumulation, predicted from the ΔpH, were as follows: 57, 0%–10%; 24, 10%–20%; 6, 20%–30%; 9, 30%–40%. Although glycolate concentrations as high as 5 mM were used in some concentration series experiments (not shown), there was no evidence of a nonsaturable component of the rapid glycolate uptake.

Dependence of Apparent K_m and V_{max} on Stromal and Medium pH. Glycolate transport proceeds in some way that is equivalent to the transport of glycolic acid. The possible mechanisms include facilitated diffusion of glycolic acid, symport of a proton with glycolate, and antiport of a hydroxyl ion and glycolate. A recent study has addressed the same choice of mechanisms for L-lactate transport by the human erythrocyte monocarboxylate carrier (De Bruijne et al., 1983). Figure 5 depicts kinetic models for these modes of transport, and Table II lists expressions for their apparent K_ms and V_{max}s. The models assume a rapid binding equilibrium of the carrier and ligands, a proton or hydroxyl to substrate ratio of 1, and a constant number of ligand binding sites. The "nonmobile" carrier models assume a constant number of ligand binding sites on each side of the membrane. The mobile carrier parameters reduce to those of the nonmobile carrier, if one assumes that return of the unloaded carrier is much faster than movement of the loaded carrier (i.e., $a_2 \gg b_1$). The kinetic parameters of the various models differ in their dependence on the cis and trans pHs during initial rate transport. For example, only the mobile carrier models of hydroxyl antiport, symport with the proton binding first, and random binding symport display any dependence of the V_{max} on the trans pH ([H_i] or [OH_i] during influx). We have performed studies on the effect of both stromal and medium pH on the kinetic parameters for glycolate uptake and efflux and will compare them to the predictions of these models.

The pH of the stroma (as measured by [^{14}C]DMO uptake) has been varied by altering the pH of the medium in which the chloroplasts were illuminated prior to centrifugation. The medium pH during transport was determined by the pH of the Percoll layers. One question of concern was whether the stromal pH would change appreciably during centrifugation through a Percoll layer of a pH different from that of the original suspending medium. Unfortunately [^{14}C]DMO equilibrates too slowly to be used to answer this question. However, an experiment that addresses this problem was performed with [1-^{14}C]glycolate as the probe of stromal pH (Figure 6). Chloroplasts were illuminated in a medium of pH 6.9 with 0.035 mM [1-^{14}C]glycolate. Their stromal pH, as measured by both [^{14}C]DMO and [1-^{14}C]glycolate uptakes, was 7.5. They were then centrifuged through 10% Percoll layers of different thicknesses, which also contained 0.035 mM [1-^{14}C]glycolate, but was pH 8.0. The apparent stromal pHs were then calculated, as if the chloroplast glycolate content were in equilibrium with the pH 8.0 medium. The glycolate content of the chloroplasts before entering the pH 8.0 medium reflected the 0.6 pH unit difference between the stroma and the pH 6.9 medium. Thus, the initial apparent stromal pH of the chloroplasts was 8.6 (0 μL of 10% Percoll, Figure 6). The apparent stromal pH fell, with increasing Percoll layer volume, reaching 7.6 for 225 μL of 10% Percoll. Thus, despite

Table II: Kinetic Parameters for Transport Models of Initial Glycolate Influx[a]

mechanism	apparent K_m	apparent V_{max}
	(A) Mobile Carrier Models	
proton symport, random binding	$\frac{K'_{s,o}K''_{H,o}[a_2K'_{H,i}(K'_{H,o}+[H_o])+a_1K'_{H,o}(K'_{H,i}+[H_i])]}{K'_{H,o}[a_2K'_{H,i}(K''_{H,o}+[H_o])+b_1[H_o](K'_{H,i}+[H_i])]}$	$\frac{a_2b_1C_TK'_{H,i}[H_o]}{a_2K'_{H,i}(K''_{H,o}+[H_o])+b_1[H_o](K'_{H,i}+[H_i])}$
proton symport, H^+ binds first	$\frac{K''_{s,o}[a_2K'_{H,i}(K'_{H,o}+[H_o])+a_1K'_{H,o}(K'_{H,i}+[H_i])]}{[H_o][a_2K'_{H,i}+b_1(K'_{H,i}+[H_i])]}$	$\frac{a_2b_1C_TK'_{H,i}}{a_2K'_{H,i}+b_1(K'_{H,i}+[H_i])}$
proton symport, S binds first	$\frac{K'_{s,o}K''_{H,o}(a_1+a_2)}{a_2(K''_{H,o}+[H_o])+b_1[H_o]}$	$\frac{a_2b_1C_T[H_o]}{a_2(K''_{H,o}+[H_o])+b_1[H_o]}$
hydroxyl antiport	$\frac{K'_{s,o}[a_2[OH_i](K_{OH,o}+[OH_o])+a_1[OH_o](K_{OH,i}+[OH_i])]}{K_{OH,o}[a_2[OH_i]+b_1(K_{OH,i}+[OH_i])]}$	$\frac{a_2b_1C_T[OH_i]}{a_2[OH_i]+b_1(K_{OH,i}+[OH_i])}$
facilitated diffusion	$\frac{K_{s,o}(a_1+a_2)}{a_2+b_1}\frac{K_a+[H_o]}{[H_o]}$	$\frac{a_2b_1C_T}{a_2+b_1}$
	(B) Nonmobile Carrier Models	
proton symport, random binding	$\frac{K'_{s,o}K''_{H,o}(K'_{H,o}+[H_o])}{K'_{H,o}(K''_{H,o}+[H_o])}$	$\frac{b_1C_T[H_o]}{K''_{H,o}+[H_o]}$
proton symport, H^+ binds first	$\frac{K''_{s,o}(K'_{H,o}+[H_o])}{[H_o]}$	b_1C_T
proton symport, S binds first	$\frac{K'_{s,o}K''_{H,o}}{K''_{H,o}+[H_o]}$	$\frac{b_1C_T[H_o]}{K''_{H,o}+[H_o]}$
hydroxyl antiport	$\frac{K_{s,o}(K_{OH,o}+[OH_o])}{K_{OH,o}}$	b_1C_T
facilitated diffusion	$\frac{K_{s,o}(K_a+[H_o])}{[H_o]}$	b_1C_T

[a] K_a is the glycolic acid dissociation constant, $10^{-3.83}$ M. Adapted from DeBruijne et al. (1983).

the 1.1 pH unit difference between the two media and despite the fact that the chloroplasts had passed through 3 times the normal volume of medium, their stromal pH had shifted by no more than 0.1 pH unit.

The dependence of the V_{max} of both influx and efflux on the trans pH can be seen in Figure 7A. This result is consistent only with the mobile carrier models for proton symport with random or proton-first binding or hydroxyl antiport. In Figure 7B, V_{max}s of influx and efflux at constant trans pH are plotted as a function of cis pH. The V_{max}s of transport in both directions seem to be independent of the cis pH. This result would eliminate the random-binding proton symport model. Despite the fact that the V_{max} vs. trans pH data were obtained at different cis pHs (pH 7.0 for influx, pH 7.5 for efflux), one would expect them to coincide in a symmetrical system. We do not know, at this point, whether this apparent asymmetry reflects a real difference in the kinetic constants for influx and efflux or is an artifact of the two different methods used for these studies.

Both the remaining mobile carrier models, symport with the proton binding first and hydroxyl antiport, predict a dependence of the K_m on the cis pH. As can be seen from Figure 8, this dependence was observed for both influx and efflux. A much weaker dependence of the K_m on the trans pH was observed for both influx and efflux. At an internal pH of 7.5, the K_ms for efflux varied from 0.40 to 0.55 mM at trans pHs from 7.1 to 8.0. At an external pH of 7.0, the K_ms for influx varied from 0.05 to 0.12 mM for trans pHs from 7.2 to 8.6.

Ability of the Glycolate Carrier To Account for Photorespiratory Carbon Fluxes. Rates of photorespiration for C_3

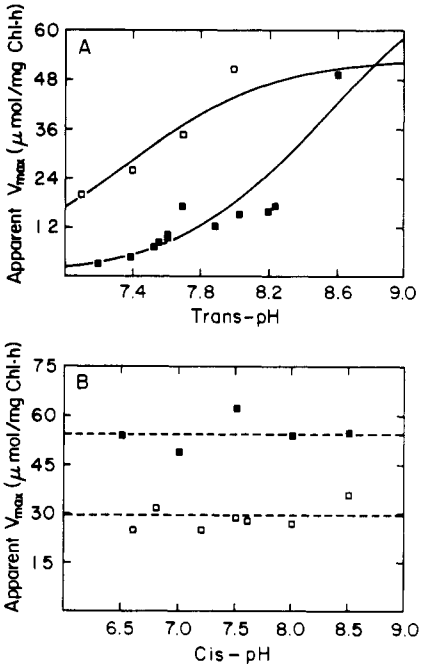

FIGURE 7: (A) Dependence of the apparent V_{max} on the trans pH. (□) V_{max}s from efflux experiments at a constant stromal pH (cis pH) of 7.5. (■) V_{max}s from influx experiments at a constant medium pH (cis pH) of 7.0. Curves were derived from the best linear fits of $1/V_{max}$ vs. $[H^+]_{trans}$. See kinetic parameters in Table II. (B) Apparent V_{max} vs. cis pH. (□) V_{max}s from efflux experiments at a constant medium pH (trans pH) of 7.6. (■) V_{max}s from influx experiments at a constant stromal pH (trans pH) of 8.6. Broken lines are the mean values of V_{max} for influx and efflux.

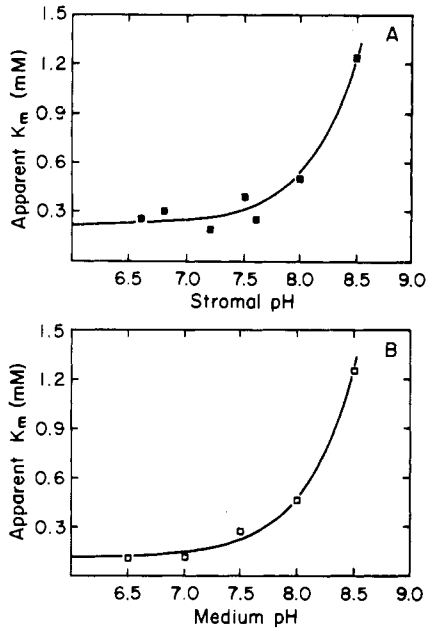

FIGURE 8: (A) Dependence of the apparent K_m for efflux on cis pH. K_ms were obtained at a constant medium pH (trans pH) of 7.6. (B) Dependence of the apparent K_m for influx on cis pH. K_ms were obtained at a constant stromal pH (trans pH) of 8.6. Curves were derived from the best linear fits of K_m vs. $1/[H^+]_{cis}$. See kinetic parameters in Table II.

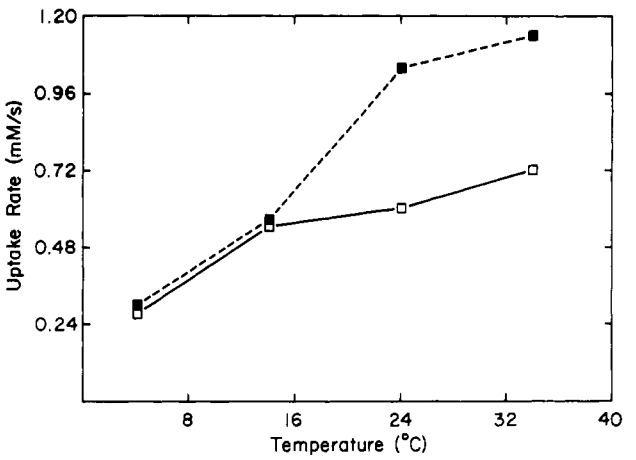

FIGURE 9: Uptake rates as a function of temperature. The [1-^{14}C]glycolate concentration was 1 mM, in a medium of pH 7.0. Tubes of type B (Figure 1) were used to obtain 2-s incubations. The composition of the silicone oil was adjusted to maintain a constant density throughout the temperature range. Versilube F-50:DC 702 ratios (by weight) were as folows: 4 °C, 8:3; 14 °C, 16:7; 24 °C, 2:1; 34 °C, 8:5. The solid line (□) represents the measured uptake rates. The broken line (■), represents the rates after correction for differences in trans pH (stromal pH). Corrections were made according to the data of Figure 7A. The stromal pHs as measured by [^{14}C]DMO uptake were as follows: 4 °C, 7.83; 14 °C, 7.84; 24 °C, 7.56; 34 °C, 7.60.

plants are estimated to be in the range of 30–45 μmol of CO_2 (mg of Chl)$^{-1}$ h^{-1} (Schnarrenberger & Fock, 1976), at 25 °C. Rates of glycolate synthesis by isolated intact chloroplasts as high as 28 μmol (mg of Chl)$^{-1}$ h^{-1} have been observed (Krause et al., 1977). At two glycolates per CO_2 evolved, an in vivo transport rate of 60–90 μmol of glycolate (mg of Chl)$^{-1}$ h^{-1} would be required to supply glycolate to the photorespiratory pathway. At 4 °C and an external pH of 7.6, we have determined the maximum glycolate efflux rate from pea chloroplasts to be about 30 μmol of (mg of Chl)$^{-1}$ h^{-1}, independent of stromal pH (Figure 7). The K_m for efflux at a stromal pH of 8.0 was 0.5 mM. Uptake rates from 2-s incubations, as a function of temperature, are plotted in Figure 9. The upper line represents the rates after correction for measured differences in the stromal pH (corrected according to data of Figure 7A). As discussed earlier, the rates at the higher temperatures are undoubtedly underestimated. In any case, the rate at 24 °C is at least 2–3-fold higher than that at 4 °C. Thus, assuming a similar temperature dependency for efflux, the maximum transporter rate under likely in vivo conditions (stromal pH 8.0, cytoplasmic pH 7.6) would be commensurate with the rate of photorespiration.

Discussion

Neither the uptake nor efflux methods described above are without flaws as sources of kinetic data on the chloroplast glycolate transporter. The assumptions involved in the use of the efflux method have been discussed. In order to improve the estimates of initial rates, an incubation time of a few hundred milliseconds would be needed. Alternative approaches would include reconstitution of the transporter into liposomes, where the transporter to internal volume ratio would be made more favorable, or partial inhibition of the transporter in organello to achieve the same result. Until such avenues can be pursued, the studies presented here can provide a useful picture of the transporter's characteristics. The methods we have outlined have achieved the shortest incubation times reported to date in a study of intact chloroplast transport. They can, in addition, be conveniently used for the processing of 100–200 microcentrifuge tubes, in a single day's experiment. Thus, they should find application to other problems in chloroplast metabolite transport.

Recently, an aqueous layer sandwiched between two oil layers was used for the study of bicarbonate transport by *Chlamydomonas* (Spalding & Ogren, 1983). A similar approach was tried with chloroplasts, but we found, when ^3H_2O and [^{14}C]sorbitol were included in an aqueous layer between two stationary oil layers, that the apparent internal volume of the chloroplast pellet was less than half of a control in which the chloroplasts were preincubated with the isotopes. It seems likely that chloroplasts that have passed through an oil layer are either damaged after entry into it or are packed together in a way that prevents equilibration with a subsequent aqueous layer. Therefore, we have used oil layers that separate the aqueous layers just until the start of centrifugation.

The symport model with the proton binding first is kinetically indistinguishable from the hydroxyl antport model (mobile carriers). Interestingly, all but these two models were eliminated by the results obtained with the erythrocyte monocarboxylate carrier (De Bruinjne et al., 1983). In this case, the authors went on to argue that, since the carrier showed no evidence of affinity for anions other than monocarboxylates, it was unlikely that hydroxyl ions would be an exception to this rule. The same argument could be advanced for the glycolate transporter. To date, we have found no evidence that ions other than the 2-hydroxymonocarboxylates lactate, glycerate, and glyoxylate (2-hydroxy when hydrated) inhibit glycolate uptake (Howitz & McCarty, 1983a,b; K. T. Howitz and R. E. McCarty, unpublished experiments). It has been noted (Deuticke, 1982; De Bruijne et al., 1983) that the random-binding proton symport model could lead to the appearance of a V_{max} independence of cis pH if $K''_H/[H_o] \ll 1 + b/a(1 + [H_i]/K'_H)$ (in a symmetrical system with $a_1 = a_2 = a$, $K'_{H,i} = K'_{H,0} = K'_H$, etc.). We have found (not shown) that chloroplasts preloaded with [^{12}C]glycolate (1 mM [^{12}C]glycolate, pH 7) took up [1-^{14}C]glycolate (0.5 mM) about 5 times faster than those that were not preloaded. This would

indicate that movement of the loaded carrier is much faster than that of the unloaded carrier ($b_2 > a_2$). Thus, the quantity b/a would be quite large, and the random-binding model still needs to be considered. Given the striking differences between the cis and trans pH dependencies of transport, it seems unlikely that nonspecific pH effects (i.e., proton or hydroxyl binding at a site not involved in the transport of these ions by the carrier) could account for our results.

Despite the quantitative discrepancies between the influx and efflux results, they are qualitatively in agreement. Both influx and efflux data showed the apparent V_{max} to be independent of cis pH and strongly dependent on trans pH. In both systems, the K_m was strongly dependent on the cis pH and weakly dependent on the trans pH. Aside from their mechanistic implications, these results indicate that both the stromal and cytoplasmic pHs could act to regulate the rate of glycolate efflux from chloroplasts and, hence, affect the intracellular levels of the various metabolites in the photorespiratory pathway.

Whether the transporter mechanism is hydroxyl antiport or a type of proton symport, it can be viewed as a use of the glycolate gradient, created by photorespiratory metabolism, to carry protons out of the stroma. The entire photorespiratory pathway represents a way of reclaiming three out of four carbon atoms lost from the Calvin cycle by the ribulose bisphosphate oxygenase reaction. Similarly, glycolate efflux could reclaim a small part of the energy loss by compensating for proton leaks into the stroma.

It is worthwhile noting that glycolate seems to be a probe of stromal pH quite comparable to DMO. Since glycolate is not metabolized by the chloroplast, it offers significant advantages over the frequently used [^{14}C]bicarbonate (Sicher, 1984).

There are at least two factors that might tend to increase the in vivo capacity of the transporter above that which we have estimated here. First, the cytoplasmic pH may be somewhat higher than 7.6. For example, cytoplasmic pH of asparagus mesophyll cells was estimated to be 7.75 (Espie & Colman, 1981). Second, we have found (unpublished results) that D-glycerate is a competitive inhibitor of glycolate uptake and can cause a counterflow of glycolate from chloroplasts. Trans acceleration of glycolate efflux by an opposing flux of glycerate would also increase the carrier's capacity under in vivo conditions.

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Registry No. Glycolic acid, 79-14-1; hydrogen ion, 12408-02-5; hydroxyl, 3352-57-6.

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