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pH Dependence and Kinetics of Glycolate Uptake by Intact Pea Chloroplasts[1]

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ABSTRACT

Experiments in which [1-^{14}C]glycolate uptake is carried out in conjunction with measurements of stromal pH indicate that only glycolic acid and not the glycolate anion is crossing the pea (*Pisum sativum* var. Progress No. 9, Agway) chloroplast envelope. This mechanism of glycolate transport appears to be too slow to account for observed photorespiratory carbon fluxes in C_3 plants.

Glycolate formation in the chloroplasts of C_3 plants most probably begins with the oxygenation of ribulose bisphosphate, catalyzed by ribulose bisphosphate carboxylase (3, 14, 15). The phosphoglycolate thus formed is then hydrolyzed to glycolate and phosphate by the chloroplast phosphoglycolate phosphatase (14, 18). There is abundant evidence that glycolate formed in this way is the source of photorespiratory carbon (4, 14, 23). As such, it must somehow cross the chloroplast envelope and reach the site of its further metabolism in the peroxisomes. Isolated, intact chloroplasts have been shown to synthesize and excrete glycolate in the light (2, 7, 12, 13, 20).

Although glycolate presumably crosses the chloroplast envelope, the mechanism by which this occurs is obscure. No evidence for mediated transport has been uncovered and there are conflicting reports bearing on the permeability of the envelope to glycolic acid and its anion (5–7, 16, 20). In swelling studies, Enser and Heber (5) found chloroplasts to be permeable to the protonated form of glycolate. Although they also observed a slow permeation by the anion, this required the driving force provided by external K^+ and valinomycin. Using vesicles formed from isolated spinach chloroplast envelopes, Poincelot (16) recorded an accumulation of glycolate to 8 mM in vesicles suspended for 20 s in a medium containing 1 mM glycolate at pH 7. Given the low pKa of glycolic acid (3.83), this result would seem to indicate rapid permeation by the anion. Recently, Takabe and Akazawa (20), reported the results of uptake studies, done at various medium pH values, on spinach chloroplasts. Despite a pH dependence of the glycolate uptake rate, they interpreted their results as indicating a significant, although possibly slower than that of the acid, permeation by the anion.

We present here the results of studies on the pH dependence of glycolate uptake and its kinetics, done in conjunction with determinations of stromal pH, in intact pea chloroplasts. These results are most consistent with a model which assumes that the pea chloroplast envelope is permeable only to the protonated form of glycolate under the conditions in these experiments.

MATERIALS AND METHODS

Plant Material. Seeds of *Pisum sativum* (var. Progress No. 9, Agway) were soaked in aerated water overnight and then planted in trays of vermiculite. The trays were placed in a growth chamber (Lab Line, No. 846) under fluorescent light, on a 12-h light/12-h dark cycle. The shoots were harvested 11 to 16 d after planting.

Chloroplast Isolation. Centrifugation through Percoll (Sigma) (21) was used for the isolation of intact chloroplasts. Fifty g of seedlings were cut into small pieces in 125 ml of a frozen slurry of one of two isolation buffers. 'Buffer A' consisted of 330 mM sorbitol, 1 mg/mg BSA, 10 μM $MgCl_2$, 20 μM EDTA, 0.5 mM Tricine, pH 6.5. 'Buffer B' contained 330 mM sorbitol, 1 mg/ml BSA, 1 mM $MgCl_2$, 2 mM EDTA, 50 mM Tricine, pH 7.9. The cut shoots in isolation buffer were ground for 3 s at low speed, in a Waring Blendor equipped with a razor blade. The brei was then filtered through 20 μM nylon mesh. Twenty-seven ml of the filtered liquid was poured into each of four, 50-ml centrifuge tubes and underlayered with 14 ml of 40% (v/v) Percoll (Sigma), 330 mM soribtol, 1 mg/ml BSA, 0.1 mM $CaCl_2$, 50 mM Hepes, pH 7.5. The tubes were centrifuged 2 min at 2°C, 3,600 rpm (2200*g*), in an International PR-2 centrifuge with a swinging bucket head. The supernatants were removed by suction and the pellets containing the intact chloroplast resuspended by gentle swirling in 330 mM sorbitol, 0.5 mM Tris-HCl, pH 7.5, hereafter referred to as 'resuspension buffer.' Chl was determined by the method of Arnon (1).

Intactness and Purity of the Chloroplast Preparations. Intactness was routinely measured by comparison of the light and ferricyanide-dependent O_2 evolution of aliquots of the suspension which had been subjected to hypotonic shock to those maintained in an isotonic medium (8). Intactness as determined by this method correlated well with results obtained on the same preparation by electron microscopy. The intactness of the preparations used for the experiments presented here ranged from 80% to 95%. The preparation procedure yielded chloroplasts essentially free of mitochondria, as measured by fumarase activity (17). The chloroplast pellet contained only 0.25% of the total catalase activity, (O_2 evolution in 10 mM H_2O_2, 10 mM sodium phosphate, [pH 7]), indicating substantial freedom from peroxisomes. O_2 evolution was measured with a Clark-type O_2 electrode model D. W., Hansatech).

Uptake Studies. The incubation solutions used for determining glycolate uptake were originally designed to follow release of glycolate from the chloroplasts after they had been allowed to accumulate it at a medium pH of 5. A second solution was added, diluting the chloroplast suspension and shifting the pH to a higher value. Results obtained in this way were found to be very similar

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to those obtained by allowing the chloroplasts to take up glycolate in the mixture of the two solutions (data not shown). Takabe and Akazawa (20) have also observed substantial symmetry between the uptake and excretion processes. Only uptake data will be presented here. The composition and pH values of the incubation solutions are as follows: all solutions contained 330 mM sorbitol, 1.3 mM $MgCl_2$, 10 mM sodium citrate, and 1.40 mM sodium glycolate. Additions made to the solutions of different pH values were: pH 5.2 or 5.3, none; pH 6.0, 14 mM Hepes; pH 7.0, 9.6 mM Tricine; pH 8.0, 20 mM Tricine. pH adjustments to the buffers used were made with HCl or NaOH. The activity of [1-^{14}C] glycolate was 2.2 μCi/ml and that of D-[1-^{3}H(N)]sorbitol was 2.1 μCi/ml. All of the above pH values and concentrations refer to those in the final incubation solutions, including the resuspended chloroplasts. Chl concentrations were from 0.16 to 0.39 mg/ml. Uptake was not found to vary with Chl concentration over this range.

Uptakes were followed by the silicone oil centrifugation technique (9). Prior to addition of the chloroplasts, the 0.4-ml polypropylene microfuge tubes contained: a bottom layer of 50 μl of 15% (v/v) glycerol, 2% (w/v) TCA; a 100-μl middle layer of silicone oil (General Electric Versilube F50:Dow Corning 702, 16:3, v/v); and a top layer of 225 μl of incubation solution. At the start of an incubation, 25 μl of chloroplasts in resuspension buffer were added and the top layer stirred with a thin glass rod. Unless otherwise indicated, incubations were done in the dark, at room temperature (20°C). At the end of the incubation time, the chloroplasts were separated from the suspending medium by centrifuging full speed for 15 s in a Coleman microfuge. The top two layers were removed by suction, the tubes cut at the former position of the silicone layers, and the pellet resuspended in the glycerol-TCA layer. The cut tubes were then recentrifuged before sampling of the glycerol-TCA for scintillation counting.

The short incubation times (15 s and less), used in the kinetic study at pH 5.30 made the above methods for initiating glycolate uptake impractical. For these experiments, tubes were prepared with a small hole bored in them, above the level to which the top layer would reach. Twenty-five μl of chloroplasts in resuspension buffer were added to 100 μl of incubation medium containing all components other than glycolate ([^{12}C] and [^{14}C]) and [^{3}H]sorbitol. After 1 min, the tubes were sealed with a cap and 125 μl of the solution containing the glycolate and [^{3}H]sorbitol was quickly injected through the hole in the tube. Timing of the incubation began when the syringe plunger was fully depressed and ended with the start of centrifugation.

Table I. *Glycolate Uptake as a Function of Medium pH*

Intact chloroplasts were incubated 1 min in 1.40 mM glycolate.

Medium pH	$[Glycolate]_{stroma}$	pH_{stroma}	Predicted $[Glycolate]_{stroma}$
A. Chloroplasts isolated in buffer A (pH 6.5). $[Glycolate]_{stroma}$ values are the mean of eight determinations on four separate preparations.			
	mM [a]		
5.3	2.5 ± 0.1		
6.0	1.2 ± 0.3		
7.0	0.25 ± 0.16		
8.0	0.0 ± 0.3		
B. Chloroplasts isolated in buffer B (pH 7.9). $[Glycolate]_{stroma}$ and pH_{stroma} values are the mean of two determinations on a single preparation.			
	mM [a]		mM
5.2	6.1 ± 2.2	5.7	4.4
6.0	2.1 ± 0.4	6.1	1.8
7.0	0.34 ± 0.03	6.4	0.35
8.0	0.18 ± 0.17	7.0	0.14

[a] Mean ± SD.

Table II. *Uptake of Glycolate by Illuminated and Unilluminated Chloroplasts*

Chloroplasts were incubated 1 min in the dark with 1.40 mM glycolate, followed by 1 min in the dark or light (6×10^5 erg cm^{-2} s^{-1}). $[Glycolate]_{stroma}$ values are the mean of two determinations on a single preparation. Chloroplasts were isolated in buffer A.

Medium pH	$[Glycolate]_{stroma}$		Increase in the Light
	Dark	Light	
	mM [a]		%
5.3	1.5 ± 0.3	1.9 ± 0.0	27
6.0	0.7 ± 0.14	1.4 ± 0.1	100
7.0	0.08 ± 0.14	0.49 ± 0.11	513
8.0	0.0 ± 0.2	0.19 ± 0.20	

[a] Mean ± SD.

In the kinetic study at pH 7, two chloroplast suspensions from a single isolation were used. Three μl of 2 mM gramicidin in ethanol was added to 600 μl of chloroplasts in resuspension buffer, yielding a gramicidin concentration of 10 μM in this suspension and 1 μM in the final incubation solution. Three μl of ethanol were added to another 600 μl of the resuspended chloroplasts for use as a control. Chl concentration as 1.77 mg/ml in the resuspension buffer.

In the experiment comparing glycolate uptake in the dark with that in the light, all incubations began with the addition, in the dark, of 25 μl of the chloroplast resuspension to the incubation medium in the microfuge tubes. After 1 min of incubation in the dark, the tubes were placed into a microfuge with a transparent top. Tubes which were not to be illuminated remained for 1 min, in the dark, before the start of centrifugation. Those which were illuminated, were exposed to 6×10^5 erg cm^{-2} s^{-1}, white light for 1 min prior to centrifugation. All other procedures were identical to those in the uptake experiments previously described.

Determination of Stromal pH. Stromal pH was measured by the uptake of [^{14}C]DMO[3] (9). The DMO concentration was 36 μM, 2 μCi/ml in the final incubation solutions. Except for the absence of [1-^{14}C]glycolate, all other conditions and methods were the same as for the glycolate uptake studies.

Solute Spaces and Medium Trapping. The internal volume of the chloroplast was determined by subtraction of the D-[U-^{14}C] sorbitol space from the $^{3}H_2O$ space (9). This was found to average 38 μl per mg of Chl in intact chloroplasts. Use of this figure automatically adjusted for the intactness of a particular preparation in stromal pH or glycolate uptake calculation. D-[U-^{14}C] Sorbitol activity was 1.0 μCi/ml and that of $^{3}H_2O$ was 3.6 μCi/ml.

Trapping of [1-^{14}C]glycolate or [^{14}C]DMO in the incubation medium centrifuged down with the chloroplasts was compensated for by having D-[1-^{3}H(N)]sorbitol included in all incubation solutions. The ^{14}C counts corresponding to the amount of medium containing the observed ^{3}H counts were subtracted from the total ^{14}C counts. The amount of trapped medium was approximately equal to the internal volume of the chloroplasts throughout these experiments.

Chemicals. Glycolic acid, gramicidin and Percoll were purchased from Sigma. D-[U-^{14}C]sorbitol, [^{14}C]DMO, and [1-^{14}C] glycolate were from Amersham and D-[1-^{3}H(N)]sorbitol from New England Nuclear.

[3] Abbreviations: [^{14}C]DMO, 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione; Ci, concentration of glycolic acid in the stroma (mM); Co, concentration of glycolic acid in the medium (mM); k_{mM}, kinetic constant for glycolic acid uptake (s^{-1}).

RESULTS AND DISCUSSION

Table IA presents glycolate uptakes after 1 min at four different medium pH values. There is a pronounced effect of medium pH on the observed uptake. Chloroplasts isolated in a more basic and highly buffered grinding medium (Table IB) show somewhat higher uptakes than those in Table IA and again a strong dependence of uptake on medium pH. Such pH effects on uptake might be accounted for by assuming that only the protonated form of the acid can penetrate the chloroplast envelope and come to equilibrium between stroma and medium. Table IB also contains the results of a stromal pH determination based on the uptake of [^{14}C]DMO. If one assumes that the protonated form of glycolate comes to an equilibrium across the envelope and equilibrates with the anion only on one side or the other, one can use the stromal and medium pH values to calculate the equilibrium stromal total glycolate concentration. This is given by:

$$[\text{glycolate}]_{\text{stroma}} = \frac{(10^{(\text{pH}_{\text{stroma}}-\text{pKa})} + 1)}{(10^{(\text{pH}_{\text{medium}}-\text{pKa})} + 1)}(1.40\ \text{mM})$$

The results of these calculations are also given in Table IB and show a pH dependence similar to that of the observed uptakes. A similar pH dependence for glycolate uptake was observed with intact spinach chloroplasts (data not shown).

The differences in glycolate uptake by chloroplasts isolated in buffers A and B (Table I) probably stemmed from differences in their stromal pH. Aside from being more highly buffered and basic, buffer B contained 1 mM $MgCl_2$, compared to 10 μM in buffer A. Buffer B is also 18 mM in K^+, (50 mM Tricine titrated to pH 7.9 with KOH). Thus, the presumably higher stromal pH of the buffer B isolated chloroplasts might be due to a Mg^{2+}-activated K^+/H^+ exchange (11). The difference in stromal pH between the two sets of chloroplasts would then be maintained when they were transferred to the Mg^{2+}-free resuspension buffer.

If the envelope membrane is permeable only to glycolic acid and not the glycolate anion, treatments which raise the stromal pH should increase uptake. Illumination of intact chloroplasts is known to increase the pH of the stroma (10, 22). Table II compares glycolate uptakes in illuminated and unilluminated chloroplasts. Illumination does in fact increase glycolate uptake.

While the strong pH dependence of glycolate uptake is suggestive of a mechanism involving permeation of the chloroplast envelope by only the acid form, only single time points were used. A model of uptake via the acid form also makes certain predictions about the kinetics of the process. Let

$$\text{Ci} = [\text{CH}_2\text{OH COOH}]_{\text{stroma}}(\text{mM})$$

and

$$\text{Co} = [\text{CH}_2\text{OH COOH}]_{\text{medium}}(\text{mM}).$$

The rate at which glycolic acid moves into the chloroplasts would be proportional to its concentration gradient, or

$$\text{Uptake rate} = k_{\text{mM}}(\text{CO} - \text{Ci}).$$

However, in order to obtain the actual rate of increase of the acid form inside the chloroplasst, the uptake rate must be multiplied by the fraction of the undissociated glycolic acid at the stromal pH. Therefore,

$$\frac{d\text{Ci}}{dt} = \frac{k_{\text{mM}}}{10^{(\text{pH}_{\text{stroma}}-\text{pKa})} + 1}(\text{Co} - \text{Ci}).$$

Integration of this expression yields:

$$-\ln(\text{Co} - \text{Ci}) = \frac{k_{\text{mM}}}{10^{(\text{pH}_{\text{stroma}}-\text{pKa})} + 1}\text{t} - \ln \text{Co}.$$

Table III presents the results of an uptake time course done at a medium pH of 5.30. The stromal pH was determined to be 5.72 by [^{14}C]DMO uptake in a 15-s incubation. This pH was used in the calculation of stromal glycolic acid concentration from the total internal glycolate. Figure 1 plots $-\ln$ (Co $-$ Ci) against time in seconds. The value of k_{mM}, the millimolar kinetic coefficient is calculated to be 6.1 s^{-1} from the slope of this line. The above data tends to rule out significant permeation of the chloroplast envelope by the glycolate anion and hence justifies the calculation of a kinetic constant for glycolic acid uptake. The chloroplasts accumulate glycolate to well above the medium concentration of 1.40 mM, seemingly tending toward the 3.6 mM equilibrium concentration which would be predicted if glycolic acid equilibrated across the envelope at the measured ΔpH.

Within 15 s and at a medium pH of 5.3, movement of the glycolate anion into the chloroplasts is apparently negligible. This does not, however, rule out measurable permeation by the anion at higher medium pH values and thus higher anion concentrations or over longer time periods. Table IV contains a time course experiment done at a medium pH of 7, over a 10-min period. In addition, one of the two chloroplast suspensions used was exposed to 10 μM gramicidin (81 μM on the final incubation solution) to observe the effects of increased proton permeability of the envelope on glycolate uptake. The lower stromal pH of the gramicidin-treated chloroplasts at 1 min is probably due to a transient membrane potential (produced by K^+ and Na^+ efflux), causing proton uptake. The potential would be eliminated by Cl^- efflux after longer incubations. The changing stromal pH values over the

Table III. *Time Course of Glycolate Uptake in a Medium pH of 5.30*

Medium glycolate was 1.40 mM and $[\text{CH}_2\text{OH COOH}]_{\text{medium}}$ = 0.0458 mM. Values are means of two determinations on a single preparation. See text for explanation of $-\ln$ (Co $-$ Ci). Chloroplasts were isolated in buffer B.

Time	$[\text{Glycolate}]_{\text{stroma}}$	$[\text{CH}_2\text{OH COOH}]_{\text{stroma}}$	$-\ln$ (Co $-$ Ci)
s	*mM*	*μM*	
1	0.66	8.4	3.29
2	0.77	9.8	3.32
5	1.2	15.3	3.49
10	2.0	25.4	3.89
15	2.6	33.1	4.37

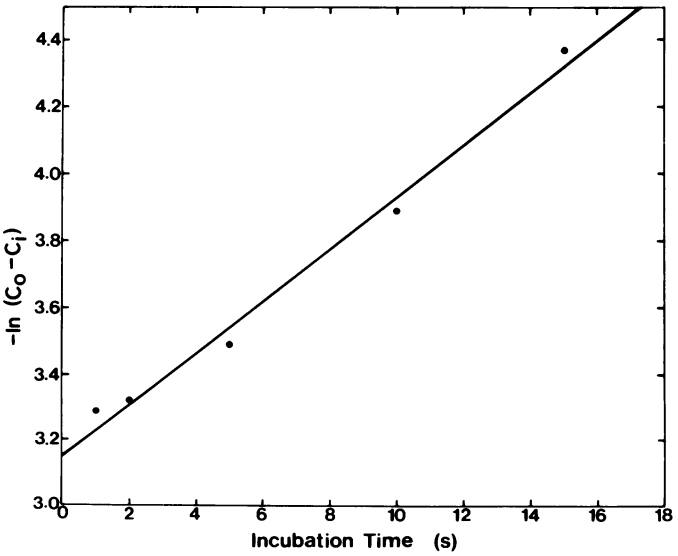

FIG. 1. Approach to equilibrium of $[\text{CH}_2\text{OH COOH}]_{\text{stroma}}$ at medium pH = 5.30. See Table III and text for data and explanation. Slope = 7.77 $\times$ 10^{-2} s^{-1}; y intercept = 3.16; correlation coefficient = 0.994.

Table IV. *Time Courses of Glycolate Uptake at a Medium pH of 7.00*

Samples with gramicidin were 10 μM in the chloroplast resuspension and 1 μM in the incubation solution. All other incubation conditions are the same as for other experiments. Uptake and stromal pH values are the mean of two determinations on a single preparation. Chloroplasts were isolated in buffer B.

Time	$[Glycolate]_{stroma}$		pH_{stroma}	
	−Gramicidin	+Gramicidin	−Gramicidin	+Gramicidin
min	*mM*			
1	0.28	0.30	6.54	6.43
3	0.24	0.57	6.64	6.67
5	0.55	0.58	6.67	6.79
7	0.64	0.72	6.75	6.84
10	0.65	0.77	6.62	6.81

course of the experiments makes calculation of k_{mM} more difficult. However, inspection of Table IV and the equations for glycolic acid uptake reveals a number of features which conform to the predictions made by a model which assumes uptake only via the acid. Neither of the chloroplast preparations comes to equilibrium with the total medium glycolate concentration of 1.4 mM. Raising the stromal pH by treatment with gramicidin has both raised the extent of glycolate uptake and lengthened its approach to equilibrium.

Rough estimations of k_{mM} can be made from the periods of the time courses over which pH is relatively constant (3–10 min, without gramicidin, 5–10 min with gramicidin). Using mean stromal pH values of 6.67 for the chloroplasts without gramicidin and 6.81 for those with, one obtains values for k_{mM} of 8.1 s^{-1} and 2.7 s^{-1}, respectively. These values bracket the 6.1 s^{-1} obtained in the experiment at pH 5.3 and all three values agree within a factor of three. The equilibrium stromal glycolate concentrations predicted from these pH values would be 0.65 mM (pH_{stroma} = 6.67) and 0.90 mM (pH_{stroma} = 6.81).

The results of the experiments presented here can best be explained by assuming that only glycolic acid and not its anion permeates the pea chloroplast envelope *in vitro*. During the course of this work, no evidence of saturation of uptake rates with increasing glycolate concentration as found (data not shown). This is consistent with previous observations on spinach chloroplasts by Takabe and Akazawa (20). Thus, simple, unmediated diffusion of glycolic acid would appear the most plausible mechanism for transport by pea chloroplsts *in vitro*.

Takabe and Akazawa (20) report pH effects on glycolate uptake rates and an uncoupler effect on the extent of uptake in intact spinach chloroplasts. They did not, however, measure stromal pH values in their experiments. It is difficult to see how uncouplers could alter the extent of uptake if, as they maintain, significant permeation by the glycolate anion occurs.

It seems somewhat doubtful that diffusion of glycolic acid across the chloroplast envelope can be the sole mechanism of glycolate transport *in vivo*. A photorespiratory rate of 40 μmol CO_2/mg Chl·h for C_3 plants (19) with a stromal volume of 38 μl/mg Chl corresponds to an increase in chloroplast glycolate concentration of 0.58 mM/s. In order to maintain a steady state of chloroplast concentration by way of glycolic acid diffusion (using the k_{mM} of 6.1 s^{-1} calculated from the data in Table III), the glycolic acid concentration in the chloroplasts would need to be

$$\frac{0.58 \text{ mM s}^{-1}}{6.1 \text{ s}^{-1}} = 0.095 \text{ mM}$$

higher than outside. At a stromal pH of 8.0 in the light (21), this is equivalent to a total glycolate concentration of 1.4 M in the stroma. Further investigation of the problem, perhaps using a system closer to *in vivo* conditions, will be required before such a physiologically improbable situation can be accepted as fact.

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