

# Evidence for a glycolate transporter in the envelope of pea chloroplasts

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Glycolate excretion by chloroplasts is essential to the process of photorespiration. Previous transport studies on intact chloroplasts have failed to find evidence for carrier-mediated transport of glycolate. The rate of glycolate uptake, when measured by a rapid silicone oil centrifugation method, saturates at high glycolate concentrations. This rapid glycolate uptake is inhibited by pretreatment of the chloroplasts with *N*-ethylmaleimide, an inhibition prevented by glycolate. Glyoxylate and glycerate inhibit glycolate uptake when present in the assay medium. These results suggest the existence of a glycolate transporter in the chloroplast envelope.

*Chloroplast  
N-ethylmaleimide inhibition*

*Photorespiration  
Glyoxylate/glycerate inhibition*

*Glycolate transport*

## 1. INTRODUCTION

Glycolate is formed in the chloroplasts of  $C_3$  plants by oxygenation of ribulose 1,5-bisphosphate, followed by the hydrolysis of the phosphoglycolate produced [1–4]. Glycolate formed in this manner is generally considered to be the major source of photorespiratory carbon [2,5,6].

Glycolate must leave the chloroplast for its metabolism in the photorespiratory pathway. However, the mechanism of this excretion is far from being completely elucidated. Two reports have appeared recently on glycolate transport by isolated, intact chloroplasts. Authors in [7] concluded that both the glycolate anion and glycolic acid can cross the envelope of spinach chloroplasts. Our results [8] indicated that only glycolic acid crosses the envelope of intact pea

chloroplasts. Neither group obtained evidence for transporter involvement in glycolate uptake or excretion. There remained a difficulty, however, in that the measured rates of glycolate transport were too low, at physiologically reasonable glycolate concentrations, to account for observed rates of photorespiration.

Here, we describe evidence that glycolate uptake by intact pea chloroplasts is transporter-mediated. When measured over a sufficiently short time, the uptake rate appears to saturate with increasing glycolate concentration. Glycolate uptake is inhibited by a pretreatment of the chloroplasts with the sulfhydryl reagent, *N*-ethylmaleimide (NEM). This inhibition can be prevented by the presence of glycolate during the pretreatment. In addition, glycolate uptake is inhibited in the presence of glyoxylate and D,L-glycerate.

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*Abbreviations:* NEM, *N*-ethylmaleimide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll

## 2. MATERIALS AND METHODS

### 2.1. Materials

Pea seeds (*Pisum sativum* var. Progress no. 9) were grown as in [8]. Glycolic acid, glyoxylic acid,

D,L-glycerate and Percoll were purchased from Sigma. D-[U- $^{14}\text{C}$ ]sorbitol and D-[1- $^3\text{H}$ (N)]sorbitol were from New England Nuclear. [1- $^{14}\text{C}$ ]glycolate was purchased from Amersham and ICN.

## 2.2. Chloroplast isolation and transport measurements

Chloroplast isolation by centrifugation through Percoll and rapid glycolate uptake measurement by silicone oil centrifugation have been described in [8]. Chloroplasts were suspended in a medium (hereafter called 'resuspension buffer') containing: 330 mM sorbitol, 50 mM HEPES-NaOH, pH 7.0, 25 mM KCl, 2 mM EDTA, 1 mM  $\text{MgCl}_2$  and 1 mM  $\text{MnCl}_2$ . Glycolate uptake assays and measurements of chloroplast internal volume were performed in this medium, except where otherwise stated. All uptake assays were done in the dark, at room temperature (18–20°C). The chloroplast internal volume ([ $^{14}\text{C}$ ]sorbitol impermeable  $^3\text{H}_2\text{O}$ -space) of the preparations used in these experiments ranged from 24–32  $\mu\text{l}/\text{mg}$  Chl and averaged 27  $\mu\text{l}/\text{mg}$  Chl. Intactness of the chloroplast preparations was checked periodically by means of ferricyanide-dependent  $\text{O}_2$  evolution before and after hypotonic lysis [9]. Intactness was always greater than 85%. Chlorophyll was determined as in [10].

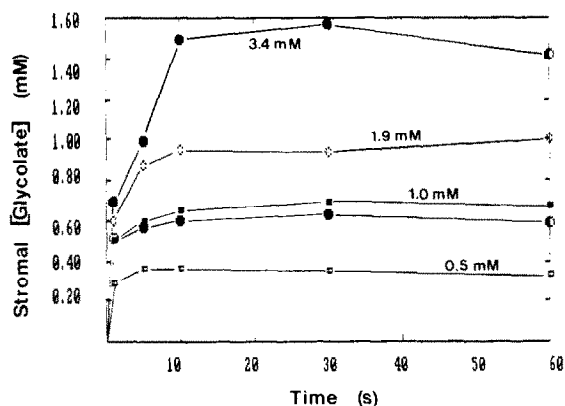


Fig. 1. Time courses of uptake at 4 glycolate concentrations. The lower 1 mM points (crosses) were obtained with chloroplasts incubated, for 1 min, in 1 mM [ $^{12}\text{C}$ ]glycolate before the addition of [ $^{14}\text{C}$ ]glycolate at time zero. The final glycolate concentration was 1 mM in both cases. All points represent the mean of two determinations.

## 2.3. Pretreatment of chloroplasts with NEM

Treatments were done on 4 aliquots of the filtered leaf homogenate (50 g of leaves) in 50-ml centrifuge tubes. NEM and glycolate in isolation buffer [8] were used for additions to the homogenate to prevent osmotic damage to the chloroplasts. Glycolate was added prior to NEM in the sample which contained both. The final concentrations of glycolate and NEM were 10 mM and 5 mM, respectively. Centrifugation of the chloroplasts through 40% Percoll [8] began 10 min after the addition of NEM and lasted 2.5 min. Each pellet was suspended in 3 ml of resuspension buffer and repelleted at  $1600 \times g$  for 1 min. These pellets were again resuspended and used for glycolate uptake assays. All of the above operations were carried out at 0–2°C.

## 3. RESULTS

### 3.1. Glycolate uptake: time courses and concentration series

Fig. 1 shows time courses of glycolate uptake, at pH 7.0, at 4 different glycolate concentrations. It is clear that there is a rapid increase in stromal glycolate concentration that is essentially complete after 10 s of incubation. This rapid phase of glycolate uptake at pH 7.0 would seem to be distinct from a much slower phase which comes to equilibrium after about 7 min of incubation [8]. Initial rates of uptake must therefore be determined in times far shorter than 10 s, a time resolution difficult to achieve by conventional silicone oil centrifugation. The modification of this method reported in [8] allows the initiation of centrifugation within 1 s after mixing the chloroplasts with incubation medium. For rate calculations, it is assumed that the chloroplasts receive 2 s additional exposure to the suspending medium after the start of centrifugation [11]. All subsequent data reported here were obtained using this protocol.

Fig. 2 shows uptake rates, at several pH's, plotted against medium glycolate concentration. Uptake is nearly unmeasurable at pH 8.2 and greatest at pH 5.0. This is consistent with the suggestion [8] that glycolic acid is the major species that crosses the envelope membrane. It is evident, at least at pH 6.3, 7.0 and 7.6, that the rate of uptake saturates at higher glycolate concentrations. We had failed to observe this saturation previously

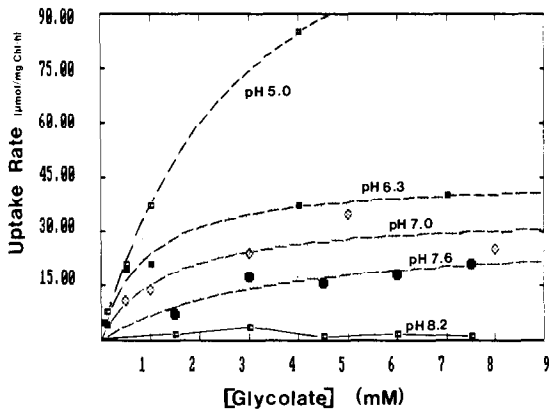


Fig. 2. Glycolate uptake rates as a function of concentration, at various pH-values. The buffers for assay at pH 5.0 and 6.3 contained 10 mM citrate in addition to the components listed in section 2. pH 7.0 points are the means of 3 determinations. All other points represent the mean of 2 determinations. The broken lines were derived from direct least squares fits to the Michaelis-Menten equation of the pH 5.0, 6.3, 7.0 and 7.6 points. The kinetic parameters  $K_m$  (mM) and  $V_{max}$  ( $\mu\text{mol}\cdot\text{mg Chl}^{-1}\cdot\text{h}^{-1}$ ) and the correlation coefficients ( $CC$ ) are: pH 5.0,  $K_m = 2.9$ ,  $V_{max} = 146$ ,  $CC = 0.998$ ; pH 6.3,  $K_m = 0.9$ ,  $V_{max} = 45$ ,  $CC = 0.991$ ; pH 7.0,  $K_m = 1.3$ ,  $V_{max} = 35$ ,  $CC = 0.893$ ; pH 7.6,  $K_m = 3.4$ ,  $V_{max} = 30$ ,  $CC = 0.915$ .

(Howitz and McCarty, unpublished) using a longer (30 s) incubation time. Authors in [7] also did not observe the saturation of uptake rates, using spinach chloroplasts and 15 s incubations at 2°C. Saturation would, of course, be expected were a transporter involved in the uptake process. To test the possibility that glycolate caused this apparent saturation in some non-transporter-dependent fashion (by, for example, altering the pH difference across the envelope) a time course of uptake was done with chloroplasts which had been preincubated with [ $^{12}\text{C}$ ]glycolate. This treatment had little effect on uptake (fig.1).

### 3.2. Inhibition of glycolate uptake by NEM

In fig.3 uptake rates at 1 mM glycolate are plotted as a function of pH. At all pH's tested, the rate of uptake is highly sensitive to inhibition by pretreatment of the chloroplasts with the sulfhydryl reagent, NEM (5 mM). This inhibition is effectively prevented by the presence of glycolate (10 mM) during the pretreatment. These results

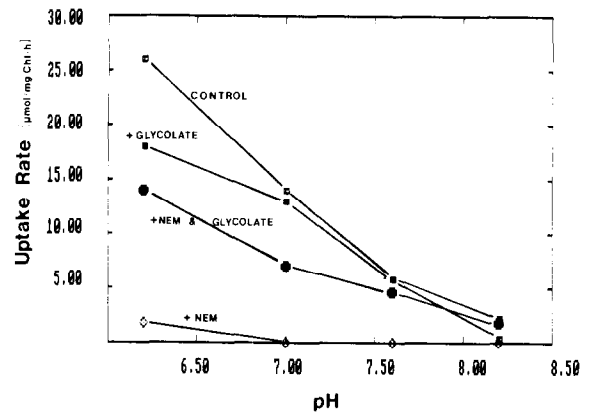


Fig. 3. Effect of NEM pretreatment of intact chloroplasts. See section 2 for details of the pretreatment procedure. All 4 chloroplast suspensions had sorbitol impermeable water spaces of  $27 \mu\text{l}/\text{mg Chl}$  after treatment. All points represent the mean of two determinations. The glycolate concentration in the uptake assays was 1 mM.

cannot be explained as resulting from damage to the integrity of the envelope membrane, since the sorbitol impermeable water space is unaffected by the NEM treatment (see legend to fig.3). It is difficult to explain the marked protection from NEM inhibition by glycolate on the basis of non-specific damage to the envelope. Glycolate and NEM do not react under the conditions of the experiment. Like the saturation of uptake rates at high

Table 1

Effect of substrates on the rate of glycolate uptake		
Additions	Uptake rate Mean $\pm$ SD ( $\mu\text{mol}\cdot\text{mg Chl}^{-1}\cdot\text{h}^{-1}$ )	% of Control
None	21 $\pm$ 4	100
[ $^{12}\text{C}$ ]Glycolate	11 $\pm$ 3	52
D,L-glycerate	12 $\pm$ 2	57
Glyoxylate	6 $\pm$ 3	30
Sodium phosphate	20 $\pm$ 2	95
Sodium acetate	18 $\pm$ 3	86
Sodium bicarbonate	17 $\pm$ 6	81

Assays were carried out at pH 7.0, in the presence of 1 mM [ $^{14}\text{C}$ ]glycolate and a 5 mM concentration of the added substrate. (The rate in the [ $^{12}\text{C}$ ]glycolate experiment is thus one sixth of the total glycolate uptake rate for that sample.) All rates represent the mean of 3 determinations

glycolate concentration, these results are consistent with transporter involvement in glycolate uptake.

### 3.3. *Inhibition of glycolate uptake by glyoxylate and D,L-glycerate*

The uptake rate of  $^{14}\text{C}$ -labeled glycolate was assayed in the presence of various other substances. Of the compounds tried, only D,L-glycerate and glyoxylate inhibited glycolate uptake appreciably (table 1). These data bear an interesting similarity to the results in [12] where it was found that D,L-glycerate uptake by spinach chloroplasts was inhibited by glycolate and glyoxylate. The specific inhibition of glycolate uptake by related substrates supports the concept that glycolate transport is carrier-mediated.

## 4. DISCUSSION

The data presented above give a strong, although preliminary, indication that the transport of glycolate is a mediated process. When determined at short incubation times, this process appears to saturate with respect to glycolate concentration, to be sensitive to NEM and to be inhibited by glyoxylate and glycerate. None of these properties would be expected if glycolate transport were to occur by free diffusion. More accurate determinations of the kinetic parameters of transport await measurement with a much shorter resolving time. Nonetheless, the maximal velocities of glycolate uptake (at the physiologically relevant pH 7.0 and 7.6), even though they may be underestimated, are much more in line with the rate of photorespiration than those estimated in [7,8].

Aside from being consistent with the existence of a glycolate transporter, the results presented here suggest several lines of further research. The

results of the NEM pretreatment experiments indicate a possible route to identifying the transporter protein by labeling it with radioactive NEM. The kinetics of the transport process, such as both uptake and the more physiologically relevant excretion, will require detailed study to ascertain whether they can account for observed rates of photorespiration. Finally, since glycolate, glyoxylate and glycerate are all substrates of the photorespiratory pathway, an investigation of any interrelationship between their mechanisms of transport across the chloroplast envelope might yield insight into the regulation of photorespiration.

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