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Simulation of the Physiological Role of Spinach Glyoxylate Reductase ($NADP^+$) in Chloroplasts

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Abstract

1. NADPH-Glyoxylate reductase was separated from hydroxypyruvate reductase and characterized at pH 7.2 and 8.0. The enzyme can work at pH 8.0; the **Km** value for glyoxylate at this pH was 0.38 mM, and the Vmax there was about 40% of that at pH 7.2. The enzyme can use both NADPH and NADH as the cofactors, and the ratio of its activities depending on these cofactors was 4.4 : 1.
2. The specific activity of the enzyme in isolated chloroplasts was 20 μmole·mg^{-1} Chl·h^{-1}, and that in the crude extract was 200 μmole·mg^{-1} Chl·h^{-1}. The discrepancy was ascribed to the leakage of the enzyme from the isolated chloroplasts and the difficulty in estimation of the enzyme activity in the crude extract because of contamination by hydroxypyruvate reductase and lactate dehydrogenase.
3. The physiological role of the enzyme was simulated in terms of the consumption of reducing equivalents and the scavenging of glyoxylate in the chloroplasts, which inhibits rubisco. According to the simulation the enzyme worked as a scavenger when the conductance of the chloroplast membrane against glyoxylate was less than 5×10^{-5} cm·sec^{-1}; otherwise it worked as a member of the shuttle of consumption of the reducing equivalents in the chloroplast.

Introduction

NADPH-glyoxylate reductase was first reported by Zelitch & Gotto[1], and Thompson & Whittingham[2] showed that the enzyme is in the chloroplast fraction, which contains also peroxisomes. Tolbert *et al.*[3] reported that the reductase is only in chloroplasts and that it has a glycolate-glyoxylate shuttle with glycolate oxidase in the peroxisomes to consume the reducing equivalents in the chloroplasts. The pool size of the glycolate and glyoxylate is, however, so small that the shuttle activity cannot be assayed by measurement of the pool size by ^{14}C-tracers of the rate of CO_2 exchange[3]. One aim of this study was to separate the enzyme from hydroxypyruvate reductase and to find whether it can work in the pH of the stroma in the light. Also, the glycolate-glyoxylate shuttle was simulated to see if it can act physiologically in consuming the reducing equivalents or scavenging glyoxylate in chloroplasts.

Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; PGA, phosphoglycerate; rubisco, ribulose 1, 5-bisphosphate carboxylase/oxygenase.

Materials and Methods

Materials Spinach, *Spinacia oleracea* L., was purchased from a local market in the winter of 1985–1986 for enzyme purification. Chloroplasts were prepared from freshly harvested spinach leaves. Tobacco, *Nicotiana tabacum* L., was grown in a greenhouse. Sunflowers, *Helianthus annuus* L., swiss chard, *Beta vulgaris,* var. *cicla,* corn, *Zea mays* 1., W64X OL43, and sugar cane, *Saccharum,* CL 41-223, were grown in fields. Mature leaves were used for the total activity measurements.

Chemicals Glyoxylate was purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and NADPH from Oriental Yeast Co. (Tokyo, Japan). The protein assay solution was purchased from Japan Bio-Rad Laboratories (Tokyo), and other chemicals of analytical reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glyoxylate solution was kept at 4°C and used within one week after the pH was adjusted to 7.2 with KOH.

Enzyme assay NADPH-glyoxylate reductase activity was assayed by the method of Tolbert *et al.*[3], with some modification. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.2, 0.2 mM NADPH, 6 mM glyoxylate, and enzyme in a total volume of 2 ml. The reaction was started by the addition of glyoxylate solution and a blank was assayed in the same way without glyoxylate. Hydroxypyruvate reductase activity was assayed with 50 mM of potassium phosphate buffer, pH 7.2, 0.2 mM of NADH, 6 mM of hydroxypyruvate, and enzyme in a total volume of 2 ml. The glyoxylate reductase activity depending on NADH was assayed by the same method as mentioned above with 6 mM glyoxylate instead of the hydroxypyruvate.

Protein and chlorophyll measurements Protein was assayed by the method of Bradford[4] with bovine gamma globulin as the main standard. Chlorophyll was measured with the method of Walker[5].

Preparation of chloroplasts Chloroplasts were prepared by the method of Takabe *et al.*[6], with some modifications. Freshly harvested leaves were cut into widths of about 1 cm width, and left for overnight at 4°C. Then they were homogenized in a Waring blender for 2 sec in 4 volumes of 50 mM Mes buffer, pH 6.2, containing 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM Na_2-EDTA, 0.5 mM KH_2PO_4, and 0.1% bovine serum albumin (BSA). The homogenate was filtered through eight layers of gauze and centrifuged at 1000 × *g* for 2 min. The precipitate was suspended with use of a brush in 50 mM Hepes buffer, pH 7.2, containing 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM Na_2-EDTA, 0.5 mM KH_2PO_4, and BSA. The suspension was centrifuged again in the same way as before and the precipitate was used as the crude chloroplast suspension. The crude chloroplasts were purified with Percoll density gradient centrifugation as follows. We prepared 10 and 90% of Percoll solutions that contained 0.5 or 4.5 g of polyethylene glycol 6000, 0.1 or 0.9 g of Ficoll 400, 0.1 or 0.9 g of BSA, and 10 or 90 ml of Percoll in a 0.33 M sorbitol solution that contained 2 mM of EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM pyrophosphate, 5 mM isoascorbate, 5 mM glutathione, and 50 mM Hepes buffer, pH 6.8. A linear gradient of the two solutions was prepared with an ISCO model 570 gradient former. Then 0.5 ml of the crude chloroplast suspension was layered onto 20 ml of gradient solution, which was centrifuged at 8,000 × *g* for 15 min, and the gradient solution was fractionated into 0.5-ml portions.

Kinetic Model

Glycolate pathway and glyoxylate shuttle Figure 1 shows the glycolate pathway with intermediates and reaction rates, involving the glycolate-glyoxylate shuttle, according to

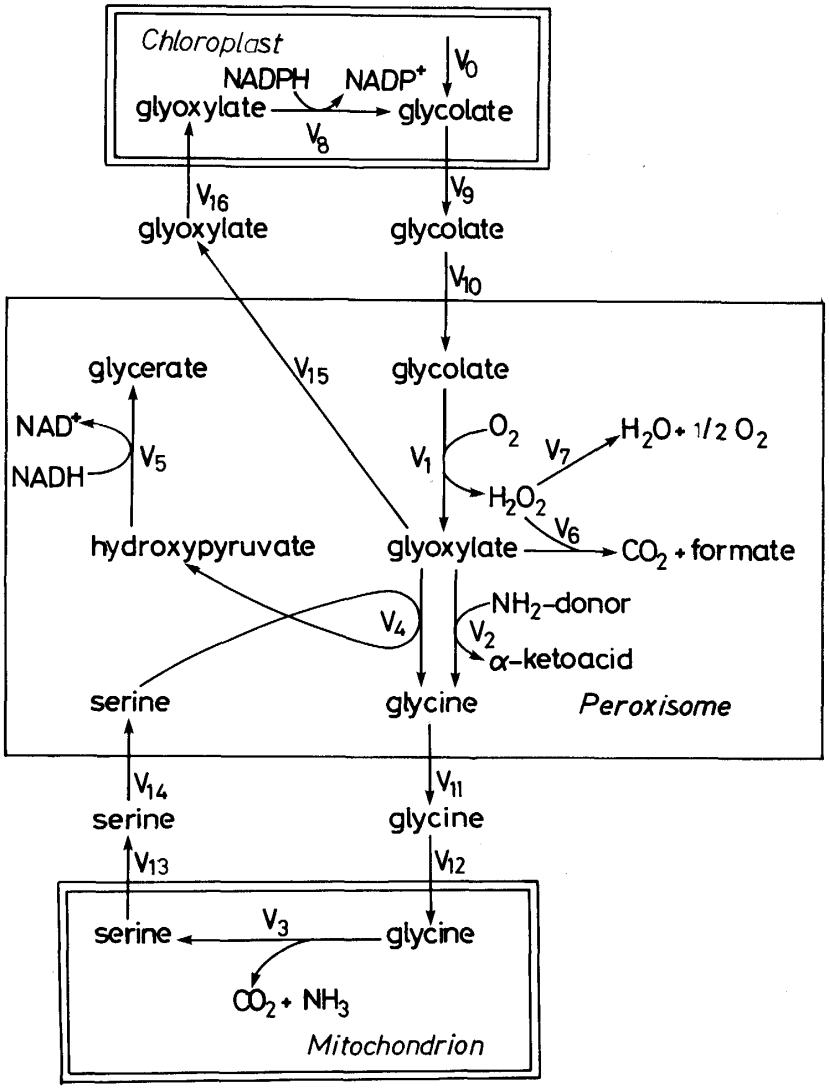


Fig. 1 Metabolic pathway of glycolate in C_3 plant cells. Stoichiometries of the reactions were taken into account in the differential equations as shown in the text. Kinetic parameters used in the simulation were as follows in the order: name of the metabolic step, maximum rate of the enzymatic reaction in μmoles·mg^{-1} Chl·h^{-1}, Km value for the substrate in mM: rate of the glycolate influx, V_0, 60[8]; glycolate oxidase, V_1, 100, 0.38 for glycolate[8,9]; alanine:glyoxylate aminotransferase, V_2, 95, 1.2 for glyoxylate[10]; conversion of glycine to serine, V_3, 94.2, 1.0 for glycine[11,12]; serine: glyoxylate transaminase, V_4, 20, 2.72 for serine, 0.15 for glyoxylate[13]; hydroxypyruvate reductase, V_5, 80, 0.12 for hydroxypyruvate[3,14]; NADPH-glyoxylate reductase, V_8, 80, 0.38 for glyoxylate (this study). k_6 is 8.17 × 10^{-3} l·μmole^{-1}·h^{-1} and k_7 is 128×10^3·h^{-1} [7]. In estimations of the maximum reaction rates, it was assumed 1.5 mg Chl = 1 g of fresh weight = 0.5 dm^2 = 3 mg nitrogen = 20 mg protein[1].

the scheme of Yokota *et al.*[7]. The velocities are as follows:

V_0 = a constant of the formation rate of glycolate;
$V_1 = V_{1\max} \times [\text{glycolate}(p)] / (\mathbf{Km}_1 + [\text{glycolate}(p)])$, the oxidation rate of glycolate in the peroxisomes;
$V_2 = V_{2\max} \times [\text{glyoxylate}(p)] / (\mathbf{Km}_2 + [\text{glyoxylate}(p)])$, the amination rate of glyoxylate to glycine in the peroxisomes;
$V_3 = V_{3\max} \times [\text{glycine}(m)] / (\mathbf{Km}_3 + [\text{glycine}(m)])$, the conversion rate of glycine to serine in the mitochondria;
$V_4 = V_{4\max} \times [\text{glyoxylate}(p)] \times [\text{serine}(p)] / (\mathbf{Km}_{4s} \times [\text{glyoxylate}(p)] + \mathbf{Km}_{4g} \times [\text{serine}(p)] + [\text{glyoxylate}(p)] \times [\text{serine}(p)])$, the deamination rate of serine to hydroxypyruvate in the peroxisomes;
$V_5 = V_{5\max} \times [\text{hydroxypyruvate}] / (\mathbf{Km}_5 + [\text{hydroxypyruvate}])$, the reduction rate of hydroxypyruvate to glycerate;
$V_6 = k_6 \times [H_2O_2] \times [\text{glyoxylate}(p)] \times \text{Vol}(p)$, the reaction rate of glyoxylate and H_2O_2;
$V_7 = k_7 \times [H_2O_2] \times \text{Vol}(p)$, the degradation rate of H_2O_2;
$V_8 = V_{8\max} \times [\text{glyoxylate}(c)] / (\mathbf{Km}_8 + [\text{glyoxylate}(c)])$, the reduction of glyoxylate to glycolate in chroloplasts;
$V_9 = \text{conductance}(c) \times ([\text{glycolate}(c)] - [\text{glycolate}(s)]) \times \text{surface}(c)$, the rate of passage of glycolate through the membrane of chloroplasts;
$V_{10} = \text{conductance}(p) \times ([\text{glycolate}(p)] - [\text{glycolate}(s)]) \times \text{surface}(p)$, the rate of passage of glycolate through the membrane of peroxisomes;
$V_{11} = \text{conductance}(p) \times ([\text{glycine}(p)] - [\text{glycine}(s)]) \times \text{surface}(p)$, the rate of passage of glycine through the membrane of peroxisomes;
$V_{12} = \text{conductance}(m) \times ([\text{glycine}(m)] - [\text{glycine}(s)]) \times \text{surface}(m)$, the rate of passage glycine through the membrane of mitochondria;
$V_{13} = \text{conductance}(m) \times ([\text{serine}(m)] - [\text{serine}(s)]) \times \text{surface}(m)$, the rate of passage of serine through the membrane of mitochondria;
$V_{14} = \text{conductance}(p) \times ([\text{serine}(p)] - [\text{serine}(s)]) \times \text{surface}(p)$, the rate of passage of serine through the membrane of peroxisomes;
$V_{15} = \text{conductance}(p) \times ([\text{glyoxylate}(p)] - [\text{glyoxylate}(s)]) \times \text{surface}(p)$, the rate of passage of glyoxylate through the membrane of peroxisomes;
$V_{16} = \text{conductance}(c) \times ([\text{glyoxylate}(c)] - [\text{glyoxylate}(s)]) \times \text{surface}(c)$, the rate of passage of glyoxylate through the membrane of chloroplasts.

In the model, it was assumed that the formation rate of glycolate was constant; that the concentration of the substrates, which were not in the glycolate pathway, was high enough to drive the glycolate cycle at the maximum rate; that glycine was converted to serine in a single reaction; and that the enzyme reaction was approximated by the Michaelis-Menten reaction, except for the amination of glyoxylate to glycine, which was expressed by the Ping-Pong Bi Bi mechanism. $\mathbf{Km}_{4s}$ and $\mathbf{Km}_{4g}$ are Michaelis constants for serine and glyoxylate in amination. The suffixes (c), (m), (p), and (s) are for chloroplasts, mitochondria, peroxisomes, and the cytosol, and Vol is for volume.

The differences in concentration across the organelle membrane were ignored in the model of Yokota *et al.*[7]. In this model, the rates of passage of intermediates through the organelles membranes were taken to be as shown in Fig. 1. Intermediates were assumed to pass through organelle membranes by simple diffusion. When the V was expressed as

μmoles/mg Chl/hr and the volume of each organelle as microliters/mg Chl, then the changes in the concentrations of intermediates in the cellular organelles were expressed as follows.

$$d\,[\text{glycolate}(c)]/dt = (V_0 + V_8 - V_9)/\text{Vol}(c)$$
$$d\,[\text{glycolate}(s)]/dt = (V_9 - V_{10})/\text{Vol}(s)$$
$$d\,[\text{glycolate}(p)]/dt = (V_{10} - V_1)/\text{Vol}(p)$$
$$d\,[\text{glyoxylate}(c)]/dt = (V_{16} - V_8)/\text{Vol}(c)$$
$$d\,[\text{glyoxylate}(s)]/dt = (V_{15} - V_{16})/\text{Vol}(s)$$
$$d\,[\text{glyoxylate}(p)]/dt = (V_1 - V_6 - V_2 - V_{15} - V_4)/\text{Vol}(p)$$
$$d\,[\text{glycine}(p)]/dt = (V_2 + V_4 - V_{11})/\text{Vol}(p)$$
$$d\,[\text{glycine}(s)]/dt = (V_{11} - V_{12})/\text{Vol}(s)$$
$$d\,[\text{glycine}(m)]/dt = (V_{12} - V_3)/\text{Vol}(s)$$
$$d\,[\text{serine}(p)]/dt = (V_{14} - V_4)/\text{Vol}(p)$$
$$d\,[\text{serine}(s)]/dt = (V_{13} - V_{14})/\text{Vol}(s)$$
$$d\,[\text{serine}(m)]/dt = (0.5 \times V_3 - V_{13})/\text{Vol}(m)$$
$$d\,[\text{hydroxypyruvate}(p)]/dt = (V_4 - V_5)/\text{Vol}(p)$$
$$d\,[\text{glycerate}(p)]/dt = V_5/\text{Vol}(p)$$
$$d\,[H_2O_2(p)]/dt = (V_1 - V_6 - V_7)/\text{Vol}(p)$$
$$d\,[\text{formate}(p)]/dt = V_6/\text{Vol}(p)$$
$$d\,[CO_2]/dt = (V_6 + 0.5 \times V_3)/\text{Vol}(p)$$
$$d\,[NADP^+(c)]/dt = V_8/\text{Vol}(c)$$

The cell parameters used were estimated as follows. According to Heldt & Sauer[22], the volume of a chloroplast is 30 $\mu l \cdot mg^{-1}$ Chl, and the cytoplasmic volume is about the same[16]. The extrachloroplast cell space is usually 5 to 10 times the osmotic chloroplast volume; we here assume it to be 225 $\mu l \cdot mg^{-1}$ Chl. Thus the volume of the vacuoles should be 195 $\mu l \cdot mg^{-1}$ Chl. Chloroplasts measured by electron micrography are spheroids with a major axis of 3.0 μm and a minor axis of 1.5 μm[17], so the volume per chloroplast was 3.5 μm^3. Therefore, the cell contained 8.6×10^9 chloroplasts $\cdot mg^{-1}$ Chl. The diameters of mitochondria and peroxisomes are 0.9 and 0.3 μm from electron micrography[17]. If we assume that these organelles are spheres, their volumes were 0.39 μm^3 and 0.014 μm^3, respectively. The numbers of chloroplasts, peroxisomes, and mitochondria were assumed here to be roughly equal, so the total volume in the cell of mitochondria and peroxisomes was 3.4 and 0.12 $\mu l \cdot mg^{-1}$ Chl, respectively. The surface area of chloroplasts, mitochondria and peroxisomes was 1.19×10^3, 2.19×10^2 and 2.43×10 $cm^2 \cdot mg^{-1}$ Chl, respectively. The radius of the cell is about 7 μm, to judge from micrography[17], so the cell volume was 1430 μm^3. Thus there are about 50 of each organelle in each cell. These differential equations were calculated by the fourth-order Runge-Kutta method.

Separation of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase

First, 100 g of spinach leaves was homogenized in a Waring blender with 150 ml of 20 mM Tris-HCl buffer, pH 7.5, and then the homogenate was filtered through eight layers of gauze. The filtrate was centrifuged at $600 \times g$ for 20 min, and the supernatant was used as the crude extract. This extract was first fractionated with $(NH_4)_2SO_4$ fractionation; the fraction of 0.3 – 0.6 $(NH_4)_2SO_4$ saturation was recovered as a precipitate by centrifugation. This fraction was dissolved and dialyzed against 20 mM Tris-HCl buffer, pH 7.5, containing 30% ethylene glycol (buffer A). The dialysate was put on a DEAE-Sepharose CL-6B column (2.5 × 25 cm) equilibrated with buffer A. Hydro-

xypyruvate reductase was first washed out with 600 ml of buffer A containing 0.1M KCl, and then the enzyme was eluted with buffer A containing 0.3 M KCl (Fig. 2). The active fraction was dialyzed against buffer A and put on an Affi-gel Blue column (1.5 X 10 cm) equilibrated with buffer A. The column was washed with 150 ml of buffer A and eluted with a linear gradient of KCl from 0 to 0.3M KCl in 200 ml of buffer A (Fig. 3). The

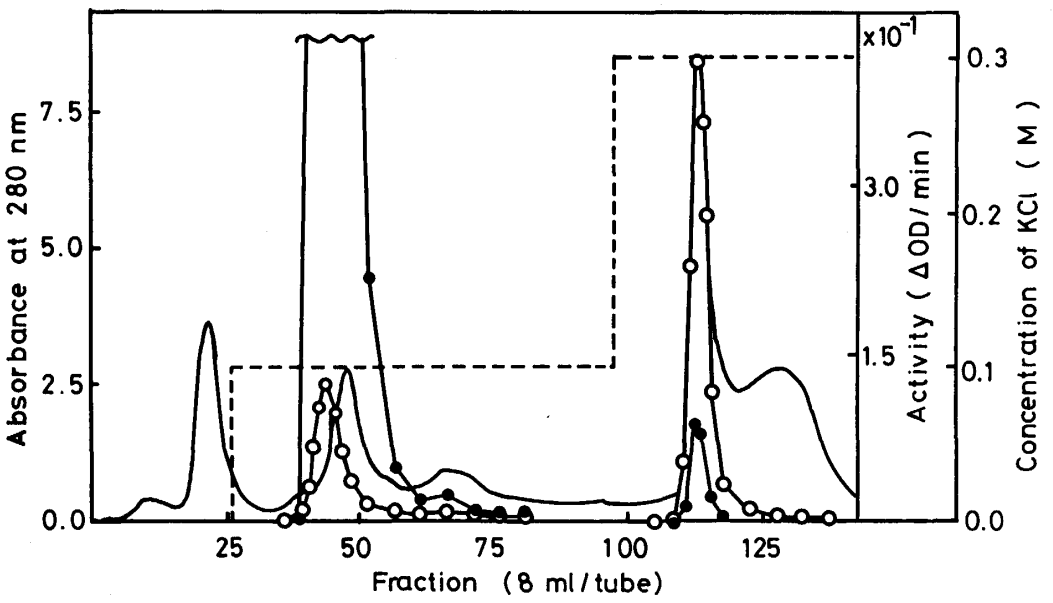


Fig. 2 Elution profile of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase from a DEAE-Sepharose CL-6B column, 2.5 X 25 cm. The solid and dashed lines indicate the absorbance at 280 nm and the KCl concentration, respectively. The open and closed circles indicate NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase, respectively.

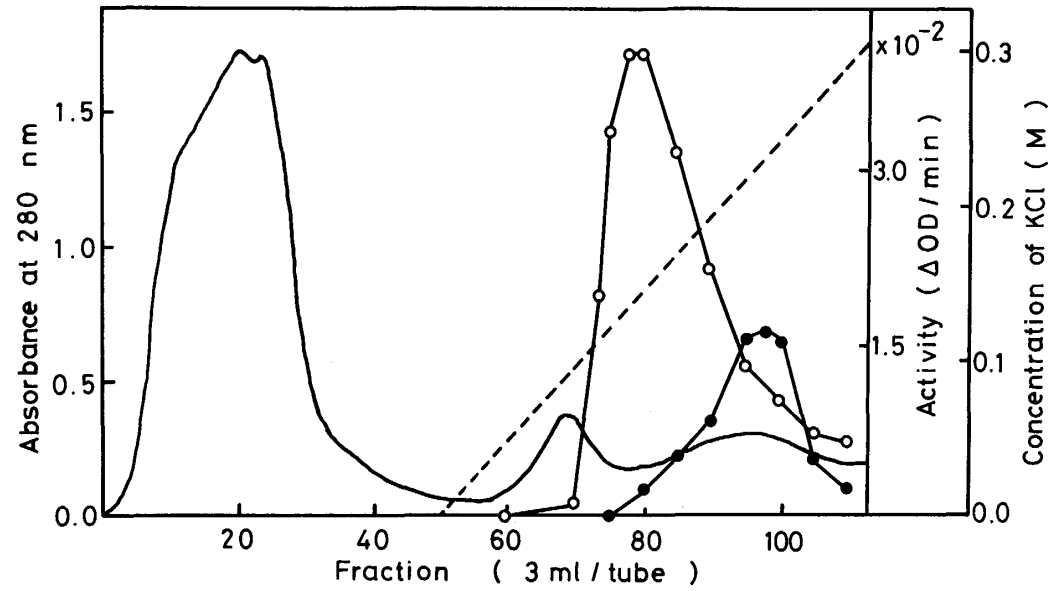


Fig. 3 Elution profile of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase from an Affi-Gel Blue column, 1.5 X 10 cm. Lines and circles are as in the legend to Fig. 2.

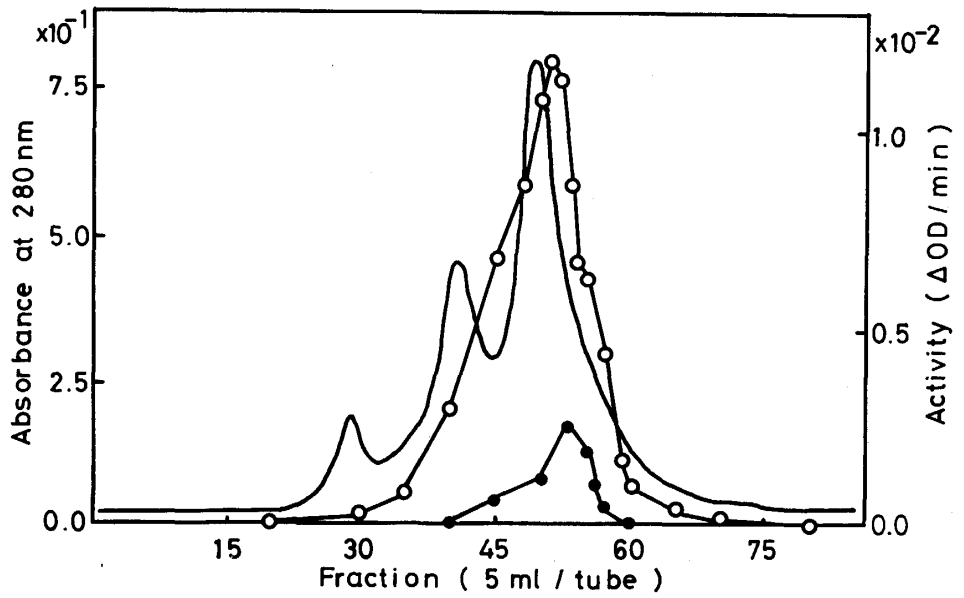


Fig. 4 Elution profile of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase from a Sephacryl S-300 column, 2 X 60 cm. Lines and circles are as in the legend to Fig. 2.

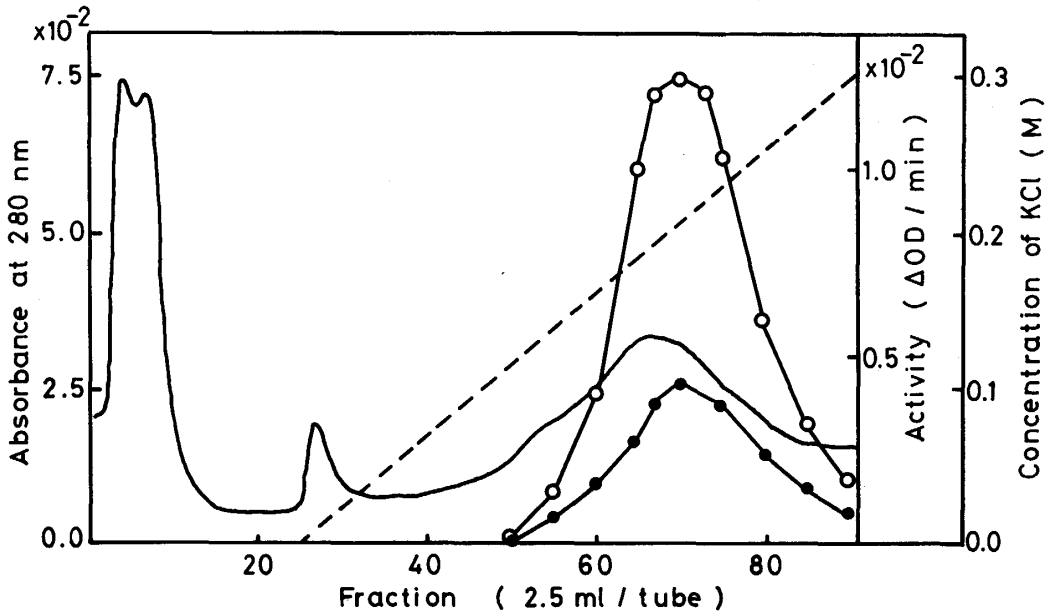


Fig. 5 Elution profile of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase from a Red Sepharose CL-6B column, 1 X 6 cm. Lines and cricles are as in the legend to Fig. 2.

active fraction was concentrated to 3.0 ml, chromatographed on a Sephacryl S-300 column (2 X 60 cm), and eluted with the same buffer (Fig. 4). The active fraction was dialyzed against the same buffer and put on a Red Sepharose CL-6B column (1 X 6 cm) equilibrated with the buffer. After the column was washed with 60 ml of the buffer, it was eluted with a linear gradient of KCl from 0 to 0.3 M in 75 ml of buffer A. The active fraction was chromatographed again on Red Sepharose CL-6B in the same way as mentioned above (Fig. 5), and the active fractions were used as the isolated NADPH-glyoxylate reductase. The separation steps are summarized in Table 1.

Table 1 Purification of NADPH-glyoxylate reductase

	Protein (mg)	Activity (unit)	Specific activity (unit/mg)	Yield (%)	Fold	NADPH/NADH
Crude extract	1452	97	0.067	100	1	0.97
$(NH_4)_2SO_4$ fractionation	877	153	0.17	158	2.6	1.4
DEAE-Sepharose CL-6B	189	74	0.39	76	5.9	3.9
Affi-Gel Blue	46	20	0.44	21	6.5	3.5
Sephacryl S-300	20	13	0.64	13	9.5	4.0
Red Sepharose CL-6B	3.4	6.8	1.9	7.0	28	4.3
Red Sepharose CL-6B	1.2	3.6	3.2	3.7	47	4.4

unit: the amount of the enzyme which oxidizes 1 μmole of NADPH per min.

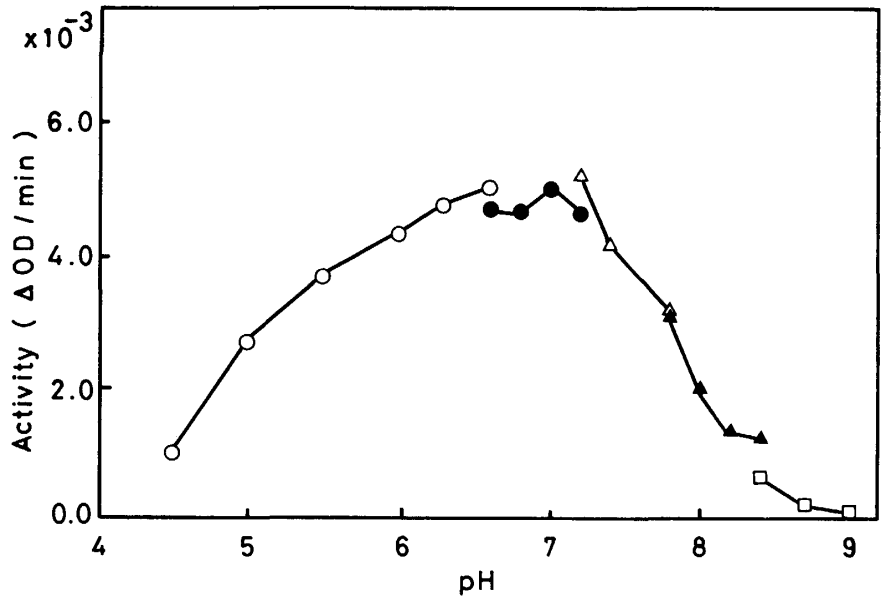


Fig. 6 Effect of pH on NADPH-glyoxylate reductase activity. Open and closed circles indicate 50 mM citrate and Pipes buffer, respectively. Open and closed triangles indicate 50 mM Hepes and Tris buffer, respectively. Open squares indicate 50 mM bicarbonate buffer.

Results

Properties of isolated enzyme The optimum pH for V_{max} was around pH 6.5 to 7.2, and the enzyme still had about 40% of its maximum activity at pH 8.0 (Fig. 6). The Km value for glyoxylate was 0.48 mM at pH 7.2 and 0.38 mM at pH 8.0, the pH of chloroplasts in the light (Fig. 7). The intermediates somewhat affected the enzyme activity (Table 2). The purified enzyme could use NADH and NADPH as cofactors, and with them, its

Table 2 Effects of metabolites on NADPH-glyoxylate reductase

	Concentration	Activity	
	(mM)	(Units × 10^2)	(%)
Control		1.71	100
ATP	1	1.90	111
ADP	1	1.90	111
AMP	1	1.93	112
$MgCl_2$	10	1.52	89
PGA	1	1.54	90
Glycolate	1	1.60	93

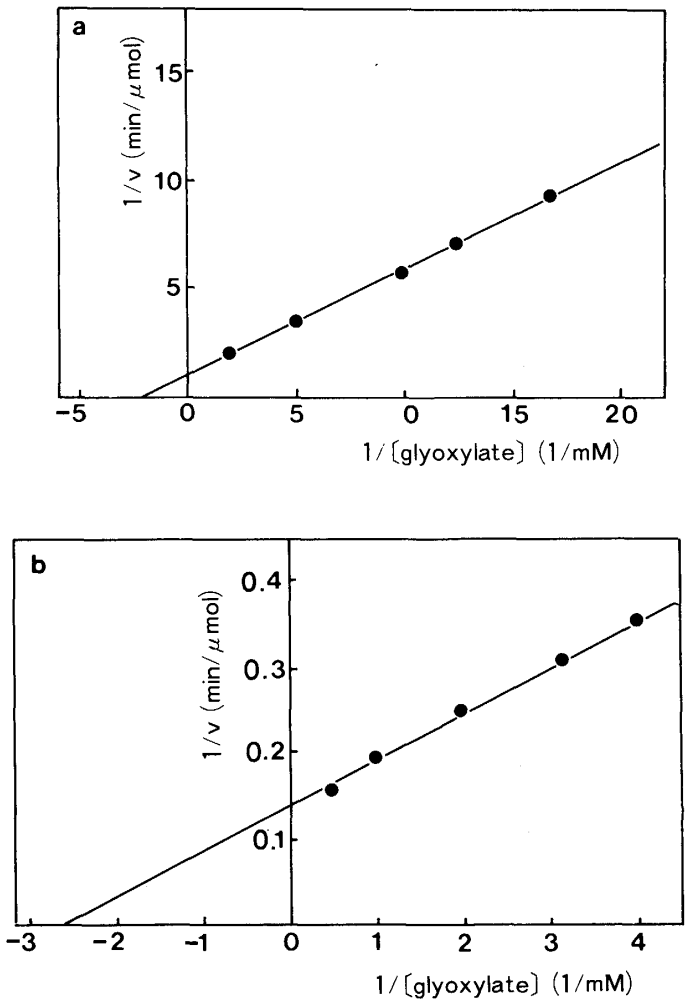
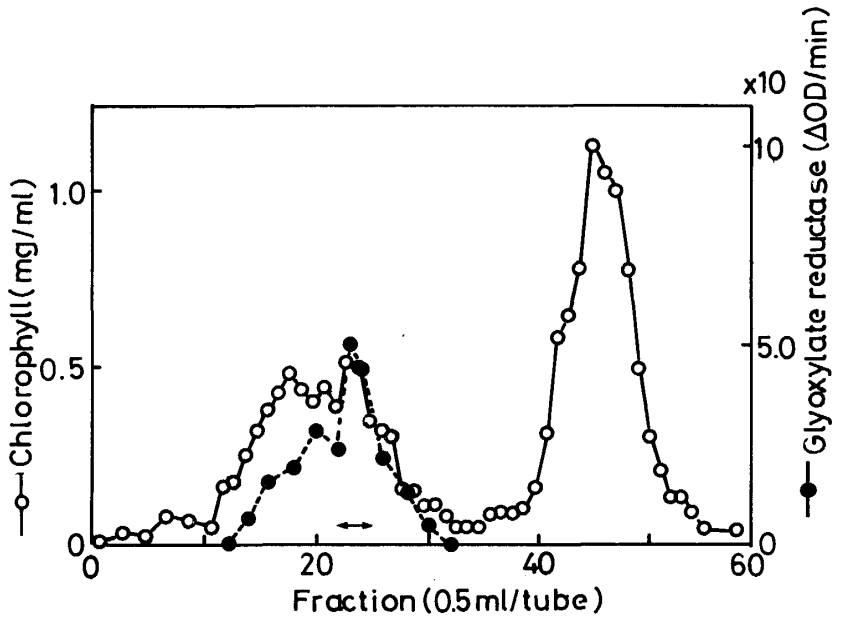


Fig. 7 Effect of glyoxylate concentration on NADPH-glyoxylate reductase. *a* and *b* show the results with pH 7.0 and 8.0, respectively. Km values were 0.48 and 0.38 mM, and V_{max} values were 3.2 and 1.3 μmole·min^{-1}·mg^{-1} protein.

activity was 0.8 and 3.2 μmole·min^{-1}·mg^{-1} protein, respectively. The enzyme acted only on glyoxylate, and did not act on hydroxypyruvate, pyruvate, oxaloacetate, glycolate, or malate.

Glyoxylate-reducing activity of chloroplasts To measure the coenzyme specificity of glyoxylate reductase and its specific activity, the enzyme activity of the isolated chloroplast fraction was assayed. The intact chloroplast fraction was treated with 0.1% Triton X-100, and the enzyme activity was measured (Fig. 8). Hydroxypyruvate reductase still contaminated the chloroplast fraction, and the maximum velocity of hydroxypyruvate reduction by the purified hydroxypyruvate reductase was 3.7-fold that for glyoxylate catalysis[18]. Therefore, the glyoxylate reductase activity with NADH was corrected by subtraction of the hydroxypyruvate reductase activity. The results showed that the activity ratio of the reductase was 1 : 3.69 on NADH and NADPH. The specific activity of NADPH-glyoxylate reductase was 20 μmoles·mg^{-1} Chl·h^{-1} in isolated chloroplasts.

Total activity of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase in some plants Whole homogenates of plant were obtained as follows for the analysis of total activity. Two grams of sample was frozen with liquid nitrogen, and homogenated with mortar and pestle, and suspended with 20 ml of 20 mM Tris-HCl buffer, pH 7.2.



substrate	cofactor	
	NADPH	NADH
glyoxylate	5.72	1.77
hydroxypyruvate	0.00	0.81 (0.22)
glyoxylate reductase	5.72 (3.69	1.55 : 1.00)

Fig. 8 Glyoxylate-reducing activity of isolated spinach chloroplast. The fractions used for glyoxylate and hydroxypyruvate reductase assays are shown by the arrow in the figure. See the text for the method of estimation of glyoxylate reductase activities on NADPH and NADH.

The suspension was centrifuged at 12,000 × *g* for 20 min. The precipitate was frozen with liquid nitrogen and extracted in the same way. The extraction was repeated until the apparent activity of the supernatant is less than 1% that of the first extract. All extracts were gathered and the whole activity was assayed. The whole homogenate contained both NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase, the first of which could not reduce hydroxypyruvate, and the second of which used both glyoxylate and hydroxypyruvate as substrate. The Vmax ratio of glyoxylate reductase with NADPH and NADH was 3.69. This ratio for the other reductase is 0.1, the Vmax ratio of the enzyme with glyoxylate and hydroxypyruvate is 1/3.7, and the Km value is 50 mM for glyoxylate[18]. The concentration in the assay medium was 6 mM; thus, the total activities of glyoxylate reductase and hydroxypyruvate reductase were estimated from the apparent activities as follows.

$$v_{Hp}' = v_{Hp} \times 1/3.7 \times 1/10 \times 1/(1 + 50/6)$$

$$v_{Gx} = v_{Gx}' - v_{Hp}'$$

v_{Hp}', the activity of hydroxypyruvate reductase on NADPH and glyoxylate

Table 3 Total activity of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase

	Glyoxylate-NADPH	OH-Pyruvate-NADH
Tobacco		
U/mg Chl	0.844 ± 0.045	29.8 ± 1.5
U/mg pro	0.0210 ± 0.0015	0.697 ± 0.038
U/g leaf	0.258 ± 0.020	8.54 ± 0.29
Sunflower		
U/mg Chl	0.134 ± 0.117	10.5 ± 5.61
U/mg pro	0.00929 ± 0.00490	0.280 ± 0.113
U/g leaf	0.461 ± 0.352	4.53 ± 2.07
Spinach		
U/mg Chl	3.35 ± 0.61	50.8 ± 2.5
U/mg pro	0.0644 ± 0.0021	1.20 ± 0.13
U/g leaf	0.995 ± 0.154	19.1 ± 4.4
Swiss chard		
U/mg Chl	6.11 ± 1.43	123 ± 35
U/mg pro	0.0414 ± 0.0047	0.811 ± 0.119
U/g leaf	0.643 ± 0.028	12.6 ± 1.3
Corn		
U/mg Chl	2.23 ± 0.44	15.3 ± 2.1
U/mg pro	0.0421 ± 0.0149	0.275 ± 0.052
U/g leaf	0.397 ± 0.136	2.56 ± 0.43
Sugar cane		
U/mg Chl	1.36 ± 0.22	7.98 ± 0.67
U/mg pro	0.0270 ± 0.0039	0.162 ± 0.023
U/g leaf	0.277 ± 0.074	1.55 ± 0.05

(U = μmoles/min, pro = protein)
The mean values are of at least three measurements.

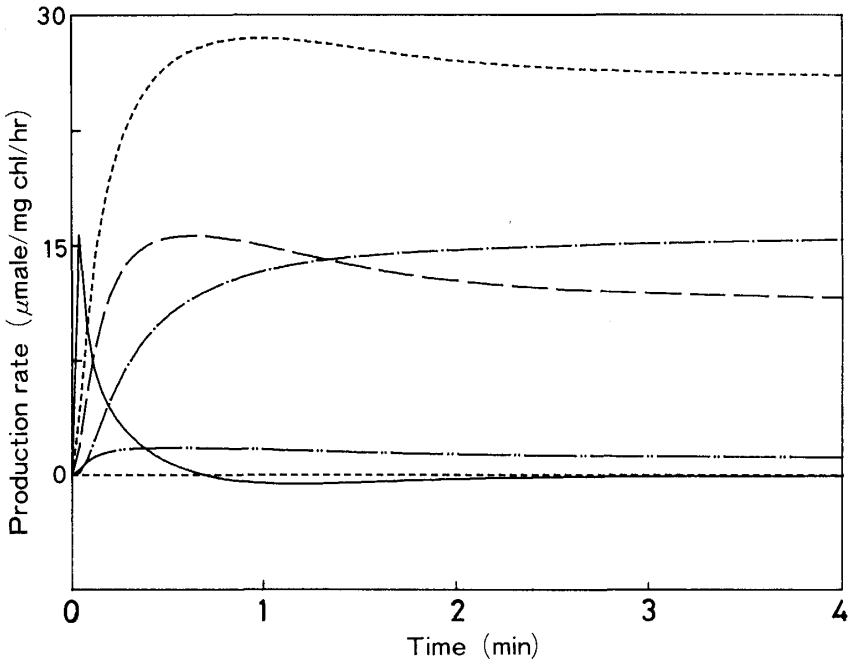


Fig. 9 Simulation of the glycolate pathway of a C_3 plant. Solid, dotted, dashed, dot dashed, and dashed with double dots lines indicate rates of glycolate production in chloroplasts, CO_2 production, NADPH oxidation, glycerate production, and serine production in peroxisomes. The conductances of the chloroplasts, peroxisomes, and mitochondria used in the simulation were 5×10^{-5}, 5×10^{-4}, and 2.5×10^{-3} cm·s^{-1}. Initial concentrations of metabolites are 0 mM.

v_{Hp}, the NADH-hydroxypyruvate reductase activity
v_{Gx}', the net NADPH-glyoxylate reductase activity
v_{Gx}, the apparent NADPH-glyoxylate reductase activity

Table 3 shows the results.

Simulation of the glycolate pathway After the first 4 min, the velocity of NADPH oxidation reached a steady state (Fig. 9). The rates of the shuttle and CO_2 liberation were 11.6 and 26.1 μmoles·mg^{-1} Chl·h^{-1} at 4 min. The photorespiratory carbon-oxidation cycle consumes 7 moles of ATP and 4 moles of NADPH for each mole of CO_2 released[21], so the rate of this cycle corresponds to 183 μmole NADPH oxidation·mg^{-1} Chl·h^{-1}. The serine concentration in the peroxisomes rose at the constant rate of 1.2 μmole·mg^{-1} Chl·h^{-1} even after 4 min. The apparent rates of other intermediate metabolisms reached a steady state, so the concentration of intermediates in all organelles was less than 2 mM at the time (results of the calculations are not shown). The steady state levels of glyoxylate and the rate of NADPH oxidation by the shuttle depended on the conductance of chloroplasts against glyoxylate (Fig. 10).

Discussion

There is some disagreement about the total activity of NADPH-glyoxylate reductase in leaves and about its cellular distribution. Intact chloroplasts prepared from spinach leaves can reduce 6.7 μmoles·mg^{-1} Chl·h^{-1} of glyoxylate[19]. Mulligan *et al.*[20] reported

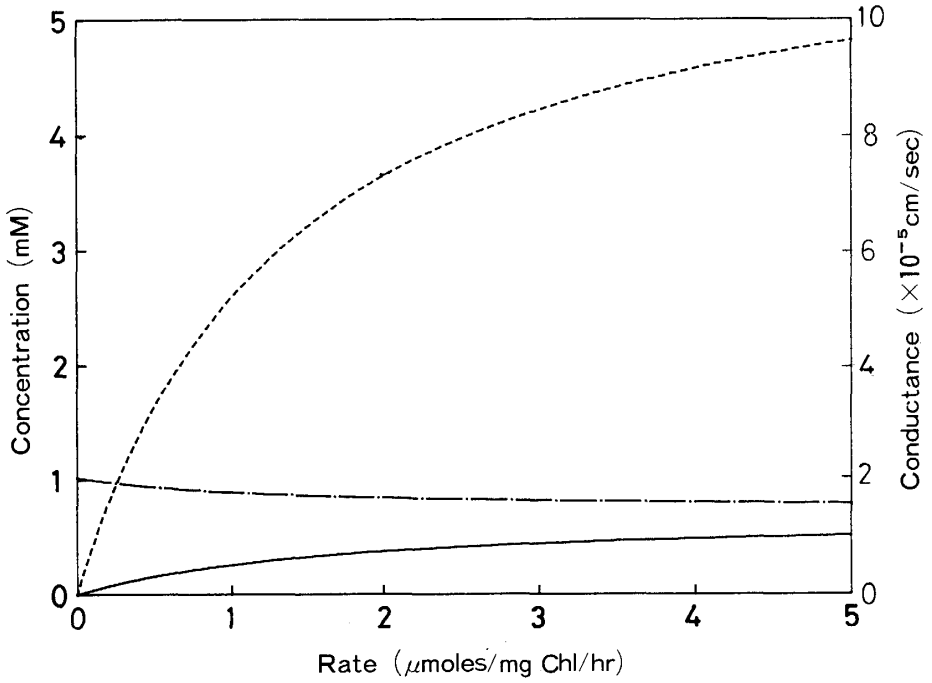


Fig. 10. Effect of the conductance of glyoxylate against chloroplasts on the role of glyoxylate reductase. Solid, and dot dashed indicate the concentrations of glyoxylate in chloroplasts, and in peroxisomes. The dotted line indicates the rate of NADPH oxidation by the glycolate-glyoxylate shuttle. All constants for the simulation were same as those in legend in figure 9 except that the conductance of glyoxylate against chloroplasts was treated as a variable. The data shown are the values after 4 min of simulation, when the oxidation of NADPH reaches a steady state.

that the rate of glyoxylate reduction is 11 μmoles·mg^{-1} Chl·h^{-1}, decreasing to 3 μmoles·mg^{-1} Chl·h^{-1} after the enzyme is solubilized. Our preparation of intact chloroplasts from spinach contained 20 μmoles·mg^{-1} Chl·h^{-1} of NADPH-glyoxylate reductase. Kleczkowski *et al.*[22] reported that crude extracts of spinach, wheat, and soy bean contain NADPH-glyoxylate activity ranging from 30 to 45 μmoles·mg^{-1} Chl·h^{-1}. The activities of crude leaf extracts from tobacco, sunflower, spinach, swiss chard, corn, and sugar cane that we report here were from 8 to 370 μmole·mg^{-1} Chl·h^{-1}. The activity we found for the crude extract of spinach was 201 μmoles·mg^{-1} Chl·h^{-1}. The discrepancies between the rates reported for chloroplasts and those for the crude extract have been ascribed to the NADPH-glyoxylate reductase being found both inside and outside the chloroplasts[22]. To our knowledge, there has been little evidence that shows that any enzyme exists among different cellular organelles. During the separation here of NADPH-glyoxylate reductase and NADH-hydroxypyruvate reductase, all of the results of chromatography showed only single peak of NADPH-glyoxylate reductase. The ammonium sulfate fractionation was done from 0.3 to 0.6 saturation, which may precipitate more isozymes that the conditions used by Kleczkowski *et al.*[22]. NADPH-glyoxylate reductase was localized almost all in the chloroplasts of spinach leaves[2,3]. We ascribed the discrepancy between the reduction rates for chloroplasts and for the crude extract to leakage of the enzyme from chloroplasts during their isolation and to the difficulty in assaying the specific reductase activity toward NADPH-glyoxylate in the

crude extract because of the presence of hydroxypyruvate reductase and lactate dehydrogenase. We concluded that there is only one species of spinach NADPH-glyoxylate reductase in these chloroplasts.

The model cell for simulation for the physiological function of the enzyme contains all of the NADPH-glyoxylate reductase in the chloroplasts and the Vmax is 40% at the optimum pH with 80 μmoles$\cdot$mg^{-1} Chl$\cdot$h^{-1}. The consumption of the reducing equivalents by the shuttle was not negligible compared with that by the glycolate pathway under the condition used for the computer simulation. In the steady state, the shuttle rate was 11 μmoles$\cdot$mg^{-1} Chl$\cdot$h^{-1}, if the glycolate input was assumed to be at the rate of 60 μmoles$\cdot$mg^{-1} Chl$\cdot$h^{-1}. The shuttle slowed when the conductance of the chloroplast membrane against glyoxylate was assumed to be 5×10^{-5} cm$\cdot$s^{-1} or less. On the same time, the glyoxylate level in the chloroplasts was decreased, but the concentration of the glyoxylate in the peroxisomes was unchanged. The results of the simulation suggested that the membrane transport of chloroplasts is important in governing the physiological role of the NADPH-glyoxylate reductase.

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