D

THESIS

Studies on the Mechanism of Light-dependent Active Transport of Pyruvate into Mesophyll Chloroplasts of C_4 Plants

by Naohiro Aoki (D3303)

Course in Biological and Environmental Sciences,
Graduate School of Science and Engineering
(Doctor's Degree Course)
SAITAMA UNIVERSITY

(Chief Advising Professor : Prof. Ryuzi Kanai)

Contents

Abstract

In C_4 photosynthesis, pyruvate uptake into mesophyll chloroplasts is an essential process. It has been known that [1] pyruvate is actively taken up into C_4 mesophyll chloroplasts in a light-dependent manner, [2] as to a substitute for the light effect, a cation (Na^+- or H^+-) jump in the medium enhances pyruvate uptake in the dark, [3] C_4 species can be divided into two groups, Na^+-type or H^+-type, due to the cation specificity, and [4] Na^+/pyruvate cotransport is operating in Na^+-type species. In this thesis, the mechanism of light-dependent active transport of pyruvate into mesophyll chloroplasts of C_4 plants was studied. It consists of two parts.

Part 1: H^+/pyruvate cotransport into mesophyll chloroplasts of H^+-type C_4 species and the role of phosphoenolpyruvate

Stromal pH of maize mesophyll chloroplasts was changed from 7 to 8 by illumination. A cotransport of pyruvate and H^+ across the envelope of maize mesophyll chloroplasts was demonstrated in the light.

1) The initial rate of H^+ consumption in the external medium on addition of pyruvate was always quantitatively related with that of [^{14}C]pyruvate uptake, namely one to one ratio of H^+ and pyruvate uptake rates. These results indicate that pyruvate uptake in the light into mesophyll chloroplasts of H^+-type C_4 species is mediated by a cotransport mechanism of H^+ and pyruvate.

2) H^+ gradient across the envelope will be the driving force of H^+/pyruvate cotransport into mesophyll chloroplasts of H^+-type C_4 species, since changes in the rate of pyruvate uptake under various pHs of the medium were closely correlated with the ΔpH formed across the envelope in the light.

3) Phosphoenolpyruvate (PEP) was transported together with H^+ in C_4

mesophyll chloroplasts via the phosphate translocator, in a manner similar to 3-phosphoglycerate transport in C_3 chloroplasts

4) Both medium alkalization and stromal acidification on pyruvate addition in illuminated maize mesophyll chloroplasts were decreased in the presence of PEP. These results show that H^+ influx accompanied with pyruvate import into illuminated mesophyll chloroplasts is compensated for with H^+ efflux together with PEP export in H^+-type C_4 species. It suggests a role of PEP in maintaining the active pyruvate uptake during the photosynthesis.

Part 2: Active transport of pyruvate in the light and a possible role of Na^+ gradient formation across the envelope in mesophyll chloroplasts of *Panicum miliaceum* L., a Na^+-type C_4 species

It has been thought without direct evidence that the driving force for Na^+/pyruvate cotransport is Na^+ gradient formed across the envelope by illumination, since Na^+-jump in the dark induced pyruvate uptake in the chloroplast. Actually, stromal pH and the amount of ATP are increased by illumination. Thus, ΔpH and/or ATP would be possible candidates of the driving force for the formation of Na^+ gradient for active pyruvate transport into mesophyll chloroplasts of Na^+-type C_4 species. Rates of pyruvate uptake in the light and dark together with or without Na^+-jump were compared under various pHs in the medium.

1) The light-dependent pyruvate uptake into mesophyll chloroplasts of *P. miliaceum* is correlated with stromal alkalization by illumination, but not with ΔpH formed across the envelope in the light.

2) Pyruvate uptake into mesophyll chloroplasts of *P. miliaceum* in the dark was not seen even in the presence of 10 mM Na^+, while the activity was induced by Na^+-jump or by illumination at external pH of 7.8. These induced

activities showed similar patterns against the concentration of Na^+. These results would indicate that illumination induces the formation of Na^+ gradient across the envelope to drive Na^+/pyruvate cotransport into mesophyll chloroplasts of Na^+-type C_4 species.

3) Pyruvate uptake due to the Na^+ gradient across the envelope occurred independently of the pH in the medium. This suggests that Na^+/H^+ antiporter may not contribute to Na^+ release from mesophyll chloroplasts of Na^+-type C_4 species.

4) Illumination and Na^+-jump had a synergistic effect on the rate of pyruvate uptake at the medium pH from 7.4 to 8.2. At this pH range, some pyruvate uptake which was induced by H^+-jump was observed even in mesophyll chloroplasts from Na^+-type C_4 species, but the rate was low compared with the effect of illumination and Na^+-jump. Besides Na^+ gradient, H^+ gradient across the envelope may contribute in some extent to pyruvate uptake in alkaline pH range.

5) ATP content in mesophyll chloroplasts did not change by Na^+-jump, negating a possible role of Na^+-ATPase in the mesophyll chloroplast envelope of *P. miliaceum*.

Abbreviations

ATP	: Adenosine-5'-triphosphate
Bicine	: N,N-Bis(2-hydroxyethyl)glycine
BSA	: Bovine serum albumin
α-CHCA	: α-Cyano-4-hydroxycinnamic acid
Chl	: Chlorophyll
CK	: Carboxykinase
p-CMS	: *p*-Chloromercuriphenylsulfonic acid
DIDS	: 4,4'-Diisothiocyano-2,2'-disulfonic acid stilbene
DMO	: 5,5'-Dimethyloxazolidine-2,4-dione
DW	: Distilled water
EDTA	: Ethylenediaminetetraacetic acid
Hepes	: 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
MCpt	: Mesophyll chloroplasts
ME	: Malic enzyme
Mersalyl	: [3-[[2-(Carboxymethoxy)benzoyl]amino]-2-methoxypropyl]-hydromercury
Mes	: 2-Morpholinoethanesulfonic acid
NAD	: Nicotinamide adenine dinucleotide
NADP	: Nicotinamide adenine dinucleotide phosphate
NBD-Cl	: 7-Chloro-4-nitrobenzoxadiazole
NEM	: N-Ethylmaleimide
OAA	: Oxaloacetic acid
PEP	: Phosphoenolpyruvic acid
2-PGA	: 2-Phosphoglyceric acid
3-PGA	: 3-Phosphoglyceric acid
Pi	: Inorganic orthophosphate
Pyr	: Pyruvic acid
SIS	: Sorbitol-impermeable space

Introduction

C_4 photosynthesis is performed by the cooperarion of two cell types in the leaf, mesophyll cells and bundle sheath cells. Figure I-1 illustrates the outline of C_4 dicarboxylic acid pathway. Atmospheric CO_2 entering mesophyll cells is rapidly converted to HCO_3^- by carbonic anhydrase in the cytosol, and is fixed to generate oxaloacetate (OAA) by phosphoenolpyruvate (PEP) carboxylase. The OAA is either reduced to malate in the chloroplast or converted to aspartate in the cytosol. These C_4 dicarboxylates are transferred to the bundle sheath cell and decarboxylated to generate CO_2 and C_3 compounds. The enzymes of decarboxylation differ in species and thus C_4 plants can be divided into three subtypes; NADP-Malic enzyme (ME) type, NAD-ME type and PEP-Carboxykinase (CK) type. The generated CO_2 is refixed by the Calvin-Benson cycle in the bundle sheath chloroplast. The C_3 compounds such as pyruvate, PEP or alanine return to the mesophyll cell and are taken up into the mesophyll chloroplast in the form of pyruvate. The pyruvate is converted to PEP by pyruvate,orthophosphate (Pi) dikinase and the PEP is released to the cytosol for the fixation of atmospheric CO_2 by PEP carboxylase (Edwards and Walker 1983). Therefore, the operation of C_4 dicarboxylic acid pathway requires both intercellular and intracellular transport of metabolites. Particularly, pyruvate uptake into mesophyll chloroplasts (MCpt) is an essential process in all subtypes of C_4 plants.

Huber and Edwards (1977a) first showed the existence of a pyruvate carrier in the envelope of MCpt of a C_4 plant, *Digitaria sanguinalis* (L.) Scop. They measured pyruvate uptake only in the dark and suggested that major mode of pyruvate uptake would be the pyruvate anion uniport, namely electrogenic transport. Thereafter, measurements of metabolite distribution

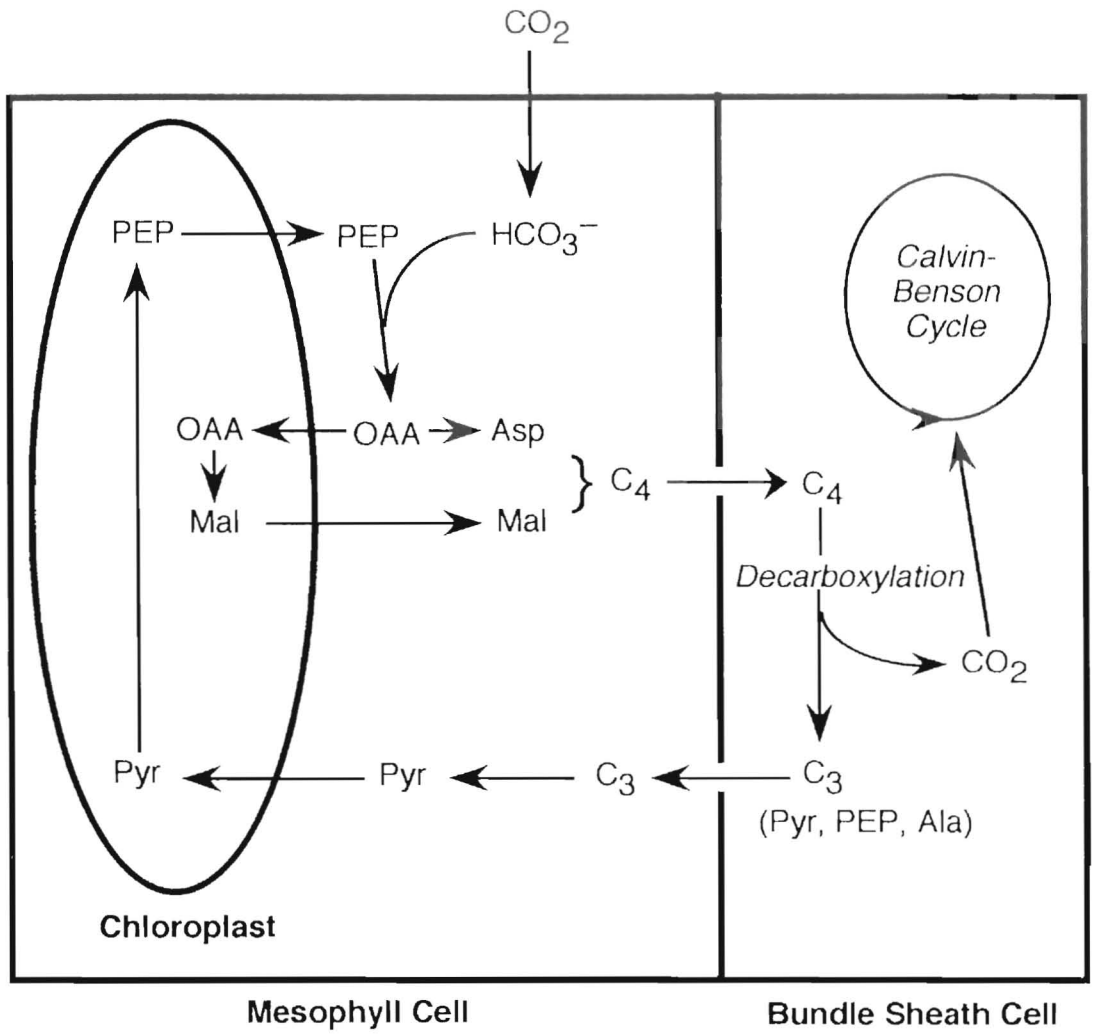


Fig. I-1. The outline of C_4 dicarboxylic acid pathway

between mesophyll and bundle sheath cells from illuminated maize leaves revealed that concentration of pyruvate in two cell types are similar (Flügge et al. 1985, Sttit and Heldt 1985). This result suggested that most of pyruvate in mesophyll cells might be accumulated in chloroplasts in the light. Actually, light-dependent active pyruvate uptake into C_4 MCpt was demonstrated in maize (Flügge et al. 1985) and *Panicum miliaceum* L. (Ohnishi and Kanai 1987a). This activity was demonstrated in MCpt of all the C_4 species tested (Aoki et al. 1992, Table I-1), but not in bundle sheath chloroplasts of *Panicum miliaceum* L. nor in C_3 chloroplasts (Ohnishi and Kanai 1987a, Table I-1).

As to a substitute for the light effect of the active pyruvate uptake, it has been shown to date that a cation (Na^+ or H^+) gradient formed across the envelope (Na^+- or H^+-jump) enhances pyruvate uptake in the dark (Ohnishi and Kanai 1987c, 1990). C_4 species can be divided into two groups, Na^+-type and H^+-type, due to the cation specificity (Aoki et al. 1992, Table I-1). Na^+/pyruvate cotransport is apparently operating in the Na^+-type species (Ohnishi et al. 1990).

In this study, I tried to elucidate the mechanism of light-dependent active pyruvate uptake into MCpt in relation to cation-jump types of C_4 species.

Table I-1. Effects of light, Na^+-jump and H^+-jump on pyruvate uptake into mesophyll chloroplasts from various C_4 and C_3 plants

Data in Aoki et al. (1992) were modified. The bold types indicate the "jump" enhancing pyruvate uptake.

Class / Family	Species	C_3 & C_4-Subtype	Rate of Pyr. Uptake (μmol / mg Chl / hr)			
			Light	Dark		
			Control	Control	Na^+-jump	H^+-jump
Monocotyledoneae						
Gramineae	*Arthraxon hispidus* (Thunb.) Makino	NADP-ME	11.9	4.2	2.7	**16.4**
	Arundinella hirta (Thunb.) C. Tanaka		14.5	3.9	3.2	**16.6**
	Coix lacryma-jobi L.		14.8	1.0	0.7	**10.1**
	C. lacryma-jobi L. cv. Mayuen		18.6	8.2	7.3	**24.7**
	Microstegium vimineum (Trin.) A. Camus		14.4	2.1	2.3	**12.3**
	Miscanthus sinensis Anderss.		11.9	1.9	2.1	**15.5**
	Saccharum officinarum L.		-	2.4	2.6	**11.6**
	S. spontaneum L. cv. Glagah		10.2	2.2	2.3	**11.6**
	S. sp. cv. Nif 4		-	2.2	2.6	**16.5**
	Sorghum bicolor (L.) Moench		16.0	6.4	6.5	**22.0**
	S. halepense (L.) Pers.		12.0	3.1	2.6	**13.0**
	Zea mays L		32.0	4.8	4.6	**24.5**
	Z. mays L. ssp. (teosinte)		38.7	5.7	5.3	**29.3**
	Cenchrus pauciflorus Benth.		10.2	1.8	**22.2**	1.5
	Digitaria cilialis (Retz.) Koel.		4.8	2.5	**12.0**	1.5
	Echinochloa crus-galli (L.) Beauv		16.9	6.7	**13.7**	3.6
	Panicum antidotale Retz.		6.8	4.6	**12.9**	4.9
	Paspalum dilatatum Poir		15.1	3.6	**22.5**	2.2
	Pennisetum alopecuroides (L.) Spreng.		13.8	4.0	**20.8**	3.9
	P. purpureum Schumach		14.3	5.7	**15.4**	5.6
	Setaria italica (L.) Beauv		16.7	7.6	**19.2**	7.0
	S. viridis (L.) Beauv.		10.8	5.1	**29.7**	0.6
	Chloris distichophylla Lag.	NAD-ME	25.2	4.2	**22.1**	4.3
	Eleusine coracana (L.) Gaertn.		9.4	5.8	**19.2**	4.2
	E. indica (L.) Gaertn.		8.5	3.4	**41.0**	5.0
	Eragrostis cilianensis (All.) Lut.		11.2	1.5	**16.7**	1.5
	E. ferruginea Beauv		11.7	9.2	**27.5**	6.0
	Panicum capillare L.		15.5	3.1	**13.9**	3.7
	P. coloratum L.		10.3	4.9	**20.9**	5.2
	P. miliaceum L.		40.8	17.8	**53.6**	11 1
	P. stapfiarum Fourc.		15.9	4.7	**27.5**	2.0
	Chloris gayana Kunth	PEP-CK	12.4	5.5	**17.0**	2.9
	Eriochloa borumensis Hackel		8.8	6.2	**22.2**	5.2
	Melinis minutiflora Beauv.		8.4	2.0	**25.0**	2.8
	Panicum maximum Jacq.		20.3	7.5	**18.6**	7.1
	P. texanum Backl.		11.2	7.1	**16.2**	9.3
	Sporobolus poiretii (R. & S.) Hintchc.		11.8	5.0	**29.1**	4.1
	Hordeum vulgare L.	C_3	6.3	5.6	7.3	6.9
Dicotyledoneae						
Amaranthaceae	*Amaranthus mangstanus* L.	NAD-ME	12.3	4.0	**20.8**	3.9
	A. retroflexus L.		-	6.6	**31.7**	8.8
	A. tricolor L.		13.1	8.5	**15.0**	8.3
Chenopodiaceae	*Kochia scoparia* (L.) Schrad	NADP-ME	13.3	6.6	**25.6**	8.8
	Salsola komarovi Iljin.		16.4	4.5	**22.7**	5.8
	Spinacia oleracea L.	C_3	9.2	8.1	9.5	6.5

Materials and Methods

Plant materials

C_4 plants used in this study were representative of the two types classified by cation specificity in enhancing pyruvate uptake into MCpt: *Zea mays* L. and *Coix lacryma-jobi* L. (H^+-type); *Panicum miliaceum* L., *Panicum maximum* Jacq., and *Setaria italica* (L.) Beauv. (Na^+-type). Seeds of these plants were sown in soil and grown on a growth bench (GB48, Conviron) under a cycle of 12 h light at 27°C / 12 h dark at 20°C, or in a growth chamber (Koito 3S-103A) under natural light supplemented with incandescent lamps, in which night / day temperatures were maintained at 25 / 30°C. Young expanding leaves (2 to 3 weeks after sowing) were used for experiments.

Isolation of mesophyll chloroplasts from leaves of C_4 plants

MCpt were isolated from mesophyll protoplasts which were obtained accoding to the method of Kanai and Edwards (1973) modified by Ohnishi and Kanai (1983). Fig. M-1 summarizes the preparation procedures. Midribs of maize leaves were removed with forceps. Leaves of C_4 plants were cut vertically across the veins into segments of 1 mm or less with a sharp razor and floated in Medium A [0.5 M sorbitol, 1 mM $CaCl_2$, 0.1% (w/v) BSA and 10 mM Mes-KOH (pH 5.5)]. Ten to twenty grams of leaf segments were incubated under illumination at 30°C for 2 h in 100 ml of Medium A containing 1% (w/v) Sumizyme C (Shin-Nihon Chemical Co. Ltd.) and 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd.). After incubation, the medium was gently discarded and the leaf segments were washed with about 150 ml of Medium B [0.5 M sucrose, 1 mM $CaCl_2$, 0.1% (w/v) BSA and 5 mM Hepes-KOH (pH 7.0)] to obtain a suspension containing mesophyll protoplasts and bundle sheath

Leaves

↓

Cut vertically across the veins into 1-mm segments or less with a sharp razor and floated in Medium A.

↓

Leaf segments

↓

Incubated in Digestion medium containing 1% Sumizyme C and 0.1% Pectolyase Y-23 at 30°C for 2 h under illumination.

Enzyme solution discarded by aspiration

(The followings are performed on ice or at 4°C.)

Washed with Medium B.

↓

Mesophyll protoplasts and Bundle sheath strands

↓

Filtrated successively through a tea strainer and a 80-μm nylon net.

Poured into Babcock flasks.

Overlaid by Medium C and then Medium D.

Centrifuged at 300 xg for 10 min.

↓

Mesophyll protoplasts at the upper interface

↓

Collected and diluted 5 times or more with Medium D.

Centrifuged at 300 xg for 5 min.

↓

Ppt.

↓

Resuspended in a chloroplast suspending medium.

The protoplasts were broken by passage through a 20-μm nylon net attached to a 5-ml syringe.

Centrifuged at 1,400 xg for 1 min.

↓

Ppt. (intact mesophyll chloroplasts)

↓

Resuspended in the suspending medium.

Fig. M-1. The preparation procedures of intact mesophyll chloroplasts from C_4 plants

strands. The following procedures were performed on ice or at 4° C. The suspension was filtrated successively through a tea strainer and a 80-μ m nylon net. Bundle sheath strands were collected on the nylon net. The filtrate was poured into two Babcock flasks up to the shoulder and overlaid by about 3 ml of Medium C [0.4 M sucrose, 0.1 M sorbitol, 1 mM $CaCl_2$, 0.1% (w/v) BSA and 5 mM Hepes-KOH (pH 7.0)] and then by about 2 ml of Medium D [0.5 M sorbitol, 1 mM $CaCl_2$, 0.1% (w/v) BSA and 5 mM Hepes-KOH (pH 7.0)]. After centrifugation at 300 xg for 10 min, mesophyll protoplasts were floated to the interface between Medium C and D. The green layer was collected with a Pasteur pipette and diluted 5 times or more with Medium D. After centrifugation at 300 xg for 5 min, the precipitate of mesophyll protoplasts was resuspended in about 5 ml of a chloroplast suspending medium [0.35 M sorbitol, 2 mM EDTA and 50 mM Hepes-KOH (pH 7.0)] and lysed by passage through a 20-μm nylon net using a 5 ml syringe. The lysate was centrifuged at 1,600 xg for 1 min. The precipitate containing intact chloroplasts was resuspended in the suspending medium. Sodium concentration in the suspending medium was reduced to as small as possible by using sodium-free KOH (minimum impurities, Nacalai Tesque) and EDTA (4H, Dojindo Laboratories). The suspending medium still contained 50 to 150 μM Na^+ ions, measured by flame photometry (Ohnishi et al. 1990). For the experiments of external pH measurement, the medium for resuspension was made buffer-free.

Silicone oil layer filtering centrifugation

Uptake of pyruvate, PEP or 5,5'-dimethyloxazolidine-2,4-dione (DMO) into MCpt was determined by a silicone oil layer filtering centrifugation method. All incubation was done on ice. Two types of the system were employed: one is a single layer system for incubation times of 7 s or longer according to Heldt

(1980), and the other is called the double layer system for incubation time of 1 to 2 s (originally developed by Howitz and McCarty 1985). Fig. M-2 summarizes the reaction systems of silicone oil centrifugation.

In the single layer system, a transparent polypropylene tube (0.4 ml, Assist) contained from the bottom, 20 μl of 1 M $HClO_4$, 70 μl of silicone oil A (Toray silicone SH550 : SH556 = 3 : 1; ρ = 1.04), and 200 μl of MCpt suspension (10 to 30 μg Chl). The reaction was started by adding 5 μl of the radioisotope mixture with a capillary glass stick. For Na^+- or H^+-jump, 2 to 5 μl of 1 M NaCl or 1 N HCl, respectively, unless stated otherwise, was added together with radioisotopes. When the tube is centrifuged with a Beckman Microfuge B for 30 s, intact chloroplasts precipitate for about 1 s through silicone oil layer to $HClO_4$ layer and the reaction is stopped. When the reaction was performed in the light, chloroplasts were illuminated for 5 to 10 min at about 300 $\mu E / m^2 / s$ on the surface of the tube. For dark assay, the reaction was performed under a dim green fluorescent light (Colored G-F, Matsushita Electric Industry Co. Ltd.) at a intensity of less than 1 $\mu E / m^2 / s$. Light intensity was measured with a photometer (LI-185B, LI-COR) attached with a LI-190SB quantum sensor.

Fifty micro-liters of the supernatant after centrifugation was mixed with 150 μl of DW and 3.5 ml of a liquid scintillator (Clear-sol I, Nacalai Tesque) in a plastic vial (Pico 'Hang-in' Vial, Packard). The tube was cut above the $HClO_4$ layer with a tube cutter. The pellet fraction was transferred to a microtube (1.5 ml, Assist) and resuspended in 300 μl of DW by vigorous shaking. After centrifugation, 200 μl of the supernatant was mixed with 3.5 ml of the liquid scintillator. Both in the supernatant and in the precipitate, radioactivities of ^{14}C and ^{3}H were measured as DPM (disintegration per minute) values with a liquid scintillation counter (LSC-1000, Aloka).

From DPM values, "Space"s were calculated as follows.

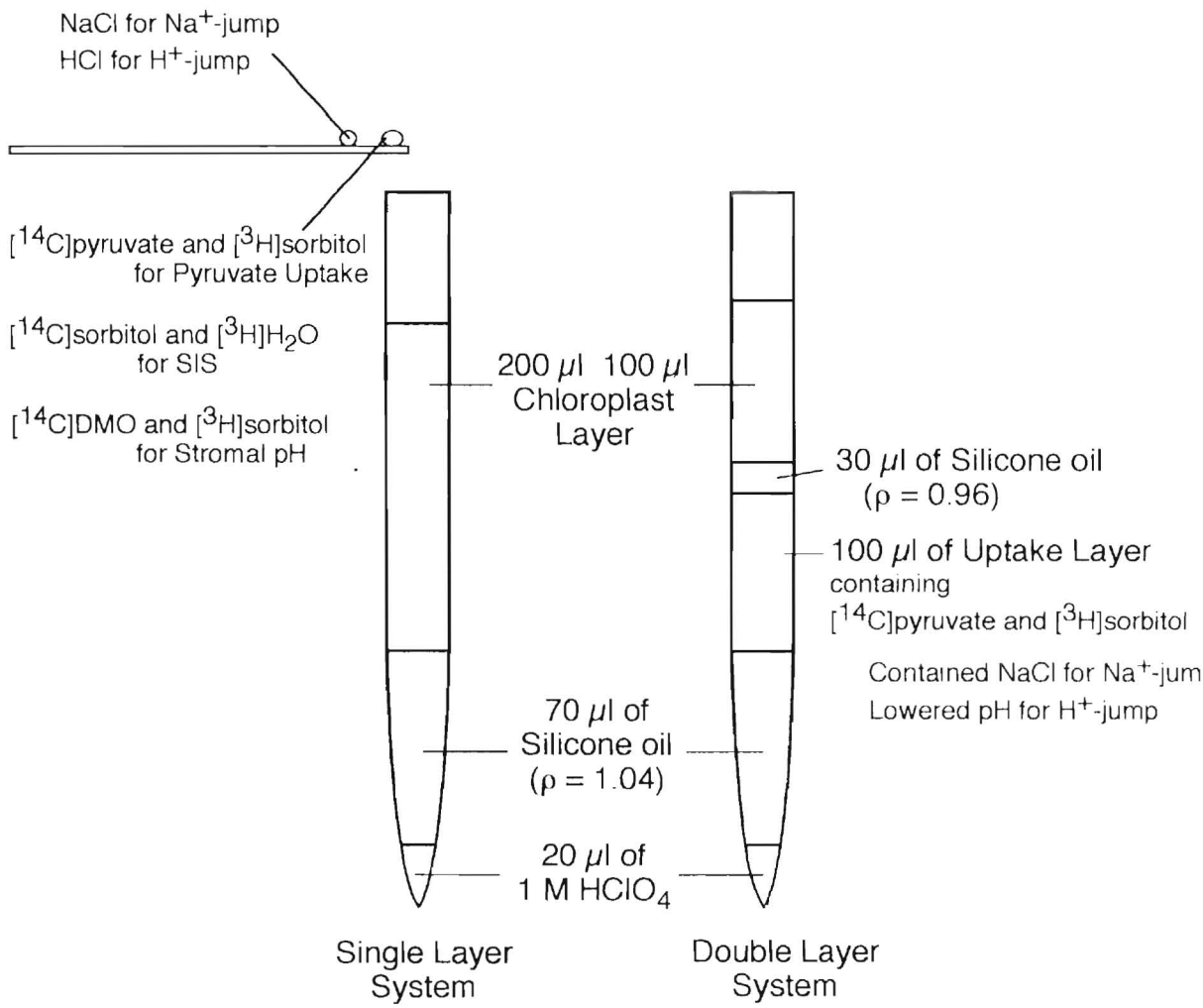


Fig. M-2. The reaction systems of silicone oil filtering centrifugation method

Space (μl / mg Chl) = [DPM_{ppt} x 320 (μl) / 200 (μl)] / [DPM_{sup} / 50 (μl)] / Chl (mg)

Determination of internal volume in chloroplast : [^{3}H]H_2O (0.3 μCi) and [^{14}C]sorbitol (0.2 μCi) were added to MCpt suspension. The inner envelope of chloroplast is impermeable to sorbitol. Thus, "Water Space" minus "Sorbitol Space" indicates internal volume in the chloroplast, called "Sorbitol-impermeable Space" (SIS, μl / mg Chl) (Heldt 1980). SIS was in the range of 15 to 25 μl / mg Chl in MCpt of all C_4 species tested.

Determination of stromal pH : Stromal pH was calculated from SIS and the uptake of a weak acid, DMO (Heldt 1980). [^{14}C]DMO (0.2 μCi, 0.5 mM) and [^{3}H]sorbitol (0.2 μCi) were added. Stromal pH was calculated as follows.

$[DMO]_{in}$ = ["DMO Space" — "Sorbitol Space"] / SIS = a

$b = 1 / (1 + 10^{pHout - pKa})$

ΔpH (In — Out) = $\log \{(a - b) / (1 - b)\}$

Stromal pH = $pH_{out} + \Delta pH$ (In — Out)

where pH_{out} is pH in the medium and pKa value of DMO is 6.64 at 4° C.

Uptake of pyruvate or PEP : [^{14}C]pyruvate (0.2 μCi) or [^{14}C]PEP (0.2 μCi) and [^{3}H]sorbitol (0.2 μCi) were added.

$[Pyr]_{in}$ (mM) = ["Pyruvate Space" — "Sorbitol Space"] x $[Pyr]_{out}$ (mM) / SIS

$[PEP]_{in}$ (mM) = ["PEP Space" — "Sorbitol Space"] x $[PEP]_{out}$ (mM) / SIS

where [Pyr or PEP]$_{in}$ is the concentration in the chloroplast and [Pyr or PEP]$_{out}$ is the concentration added to the chloroplast suspension.

In the double layer system, the tube contained from the bottom, 20 μl 1 M $HClO_4$, 70 μl of silicone oil A, 100 μl of uptake layer of the suspending medium, 30 μl of silicone oil B (Toray silicone SH556; ρ = 0.96), and 100 μl of MCpt suspension (15 to 25 μg Chl). The uptake layer contained radioisotopes, [^{14}C]pyruvate (0.1 μCi) or [^{14}C]PEP (0.1 μCi) and [^{3}H]sorbitol (0.1 μCi), and half

of its sorbitol being replaced by sucrose, and consequently the layer was heavier than the chloroplast layer but still lighter than the lower silicone oil layer. When the tube is centrifuged with a microfuge, the upper oil layer is floated to the top and intact MCpt pass through the uptake layer, react with radioisotopes for 1 to 2 s and precipitate to the bottom of the tube. Radioactivities of ^{14}C and ^{3}H, both in the uptake layer and in the precipitate, were measured as described above. The initial rate (V) of pyruvate (or PEP) uptake into MCpt was calculated as follows.

V (μmol / mg Chl / h) = ["Pyruvate Space" — "Sorbitol Space"]

x $[Pyr]_{out}$ (mM) / reaction time (h) / Chl (mg)

Reaction time in the double layer system estimated previously was 1 s. After February in 1994, calculated rates of pyruvate uptake became too high, due to declining of the acceleration power of the microfuge. Indeed, the reaction time was re-estimated to be 2 s as shown in Fig. M-3. The corrected value was adopted, thereafter.

Measurement of pH changes in the medium

A pH electrode (ORION 91-03, 6 mm diameter) connected to a pH meter (ORION 611) was inserted into the reaction vessel of an O_2 electrode (Rank Brothers, King's Lynn, UK, 15 mm inner diameter, 7 ml) and pH of chloroplast suspention was monitored with a recorder. Light intensity was 500 μE / m^2 / s on the surface of the vessel. Temperature was maintained at 5 to 6°C by water circulation.

Mesophyll chloroplast suspension (3.0 ml, 100 to 400 μg Chl / ml) was preincubated in the reaction vessel, with inhibitors when needed, for a few minutes until the pH of the external medium was stabilized, illuminated for about 5 min, and then, 10 μl of 300 mM pyruvate or 150 mM PEP was added.

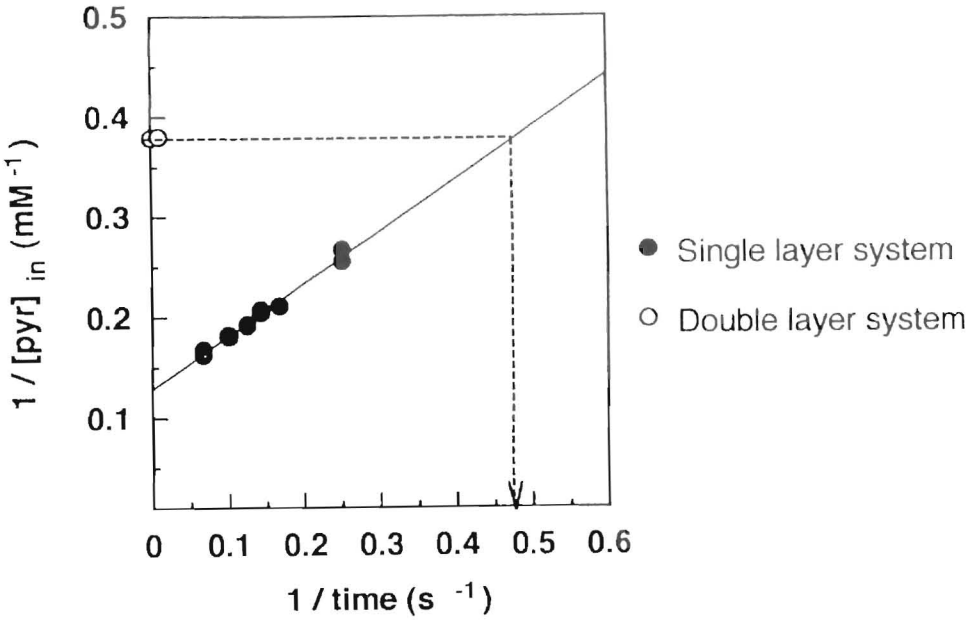


Fig. M-3. Determination of the reaction time in the double layer system

Double reciprocal plots of pyruvate uptake into maize MCpt on reaction time were obtained by the single layer system (●). From this plots (●) and $[pyr]_{in}$ obtained by the double layer system (o), the reaction time in the double layer system was estimated to be 2.03 s in this experiment.

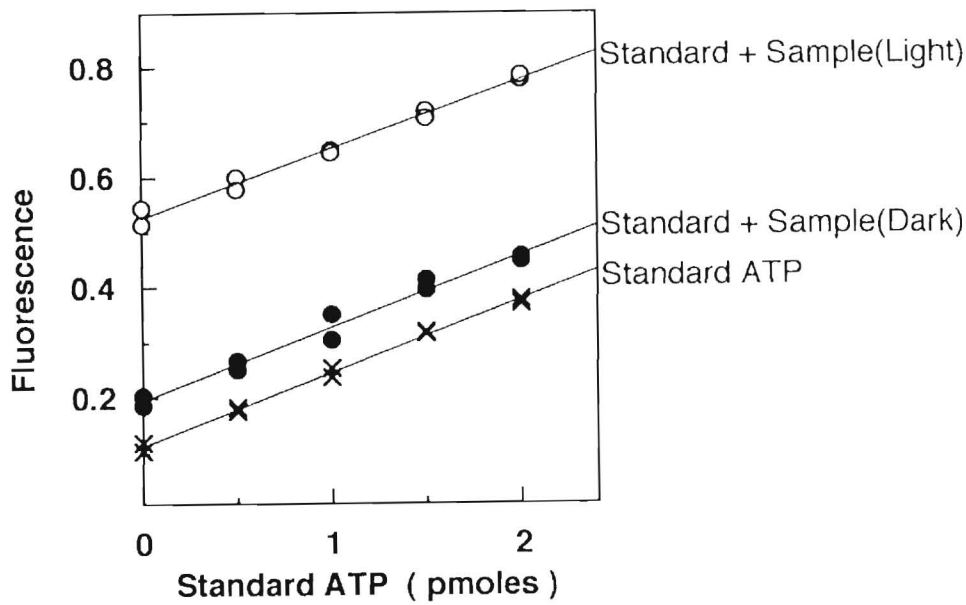


Fig. M-4. Calibration curves for the quantification of ATP

This calibration curve is made in parallel with the measurement of ATP content in the sample.

To check the sensitivity of our pH measuring system, 3-phosphoglycerate (3-PGA, 0.5 mM at final concentration) was routinely added after pyruvate and PEP addition in the experiments. Potassium salts of pyruvate, PEP and 3-PGA in neutral pH were used in these experiments.

Determination of ATP content

Samples for the determination of ATP content of the chloroplast were obtained from the precipitate from the single layer system of silicone oil centrifugation. The chloroplast precipitate in 20 μ l of 1 M $HClO_4$ was neutralized by adding 200 μl of 0.1 N KOH and centrifuged to remove $KClO_4$. The supernatant was diluted to 10 times with DW and used for determination of ATP by a luciferin-luciferase method (Strehler 1974, Kobayashi et al. 1979) using an ATP photometer (Monolight 401, Analytical Luminescence Laboratory Inc.). Firefly lanterns (50 mg, Sigma) were homogenized on ice with a pestle and a mortar in 5 ml of Buffer A [40 mM $MgSO_4$ and 100 mM Na-arsenate-H_2SO_4 (pH 7.2)]. The homogenate was centrifuged at 25,000 xg for 30 min. The supernatant was diluted 5 times with Buffer A and stored at room temperature for 30 min before use. The enzyme preparation (100 μl) was injected with a syringe of the photometer into a reaction cuvette containing 300 μl of Buffer B [10 mM $MgSO_4$, 1 mM EDTA and 30 mM Hepes-KOH (pH 7.8)] and 100 μl of an ATP mixture. The ATP mixture consisted of 20 μl of the sample, 50 nM ATP (0 to 40 μl) and DW. The luminescence from 5 s after the injection for 15 s was automatically measured. Calibration was done with known amounts of ATP, as shown in Fig. M-4. The calibration curve was linear in the range of ATP content of the sample.

Determination of chlorophyll content

Chlorophyll (Chl) content of chloroplast preparation was determined spectrophotometrically in 96% (v/v) ethanol by the method of Wintermans and De Mots (1965). Chl was extracted for more than 3 h at 4°C in the dark. After centrifugation at 1,600 xg for 5 min, A_{665}, A_{654} and A_{649} in the supernatant was measured using A_{730} as a blank.

$$\text{Chl a+b } (\mu g / ml) = 6.10 \times A_{665} + 20.04 \times A_{649} \quad (1)$$

$$= A_{654} \times 1000 / 39.8 \quad (2)$$

The wavelength of spectrophotometer (UV-240, Shimazu) was corrected by seeking the wavelength which makes the smallest difference between equations 1 and 2.

Chemicals and radiochemicals

[1-^{14}C]Pyruvic acid was obtained from Amersham or Du Pont. When [^{14}C]Pyr was a sodium-salt, Na^+ is exchanged with H^+ by a batch method, as follows; [^{14}C]Pyr in DW (300 μl) was mixed with 15 mg of a cation exchange resin (AR 50W-X8, 200-400 mesh, Bio-Rad), stirred, centrifuged, and the supernatant was used for assay.

[2-^{14}C]DMO and D-[1-^{3}H]sorbitol were obtained from American Radio-labeled Chemicals Inc. (ARC), D-[U-^{14}C]sorbitol was from Amersham or ARC, [1-^{14}C]PEP from Amersham, and $^{3}H_2O$ from New England Nuclear.

Mersalyl and *p*CMS were obtained from Sigma, DIDS and NBD-Cl from Dojindo Laboratories, NEM was from Wako, and α-CHCA from Aldrich.

Part 1

H^+/pyruvate cotransport into mesophyll chloroplasts of H^+-type C_4 species and the role of phosphoenolpyruvate

In this study to clarify the effect of light on pyruvate uptake in C_4 MCpt, a jump on Na^+ or H^+ concentration of the medium substituted for the light effect. As described in the Introduction, Na^+/pyruvate cotransport was reported in MCpt of *P. miliaceum*, a Na^+-type C_4 species (Ohnishi and Kanai 1990), but the mechanism of H^+-type C_4 species remained to be studied. Firstly, I demonstrated H^+/pyruvate cotransport into MCpt of maize, a H^+-type C_4 species by detecting H^+ uptake accompanied with pyruvate uptake. Secondly, I thought a role of PEP in maintaining the active transport of pyruvate in the light. PEP is known to be transported via the Pi translocator in C_4 MCpt (Huber and Edwards 1977b, Gross et al. 1990). Since the Pi translocator in C_3 chloroplasts transports divalent anions such as triose phosphates and Pi, 3-PGA, which is mainly a trivalent anion in a physiological pH range (pK_{a3} = 7.1), is transported together with H^+ (Fliege et al. 1978). Because of similar pK_a values of PEP (pK_a = 3.5, 6.38 (Jencks and Regenstein 1968)), I assumed that PEP transport in C_4 MCpt could also be accompanied with H^+ transport. Thus, I investigated (i) H^+ flux into MCpt on the addition of PEP in two types of C_4 plants and (ii) the effects of PEP on pyruvate-induced H^+ flux in MCpt of maize, a H^+-type C_4 species.

Results

pH changes in the external medium induced by the addition of pyruvate and 3-PGA in mesophyll chloroplasts from C_4 species

Using a pH electrode, pH changes in the external medium of MCpt from various C_4 plants were measured by adding substrates, such as pyruvate and 3-PGA. 3-PGA-induced H^+ consumption was used as a control. In all C_4 MCpt we have tested, medium alkalization was observed on addition of 3-PGA in both light and dark (Figs. P1-1 and -2). The results indicate that our pH measuring system was sensitive enough for detecting H^+ flux across the envelope accompanied with metabolite transport.

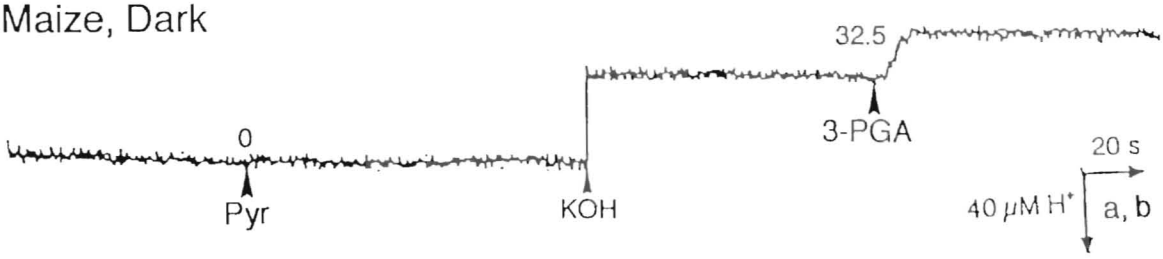
When pyruvate was added to the suspension of MCpt from H^+-type species, the medium was alkalized for 5 to 10 s in the light (Figs. P1-1b and -1c), while no or very small signal of alkalization was observed in the dark (Fig. P1-1a).

In all Na^+-type species, namely, *Panicum miliaceum* (NAD-ME C_4-subtype; Fig. P1-2a), *Setaria italica* (NADP-ME C_4-subtype; Fig. P1-2b and -2c) and *P. maximum* (PEP-CK C_4-subtype; Fig. P1-2d), no pyruvate-induced alkalization of the medium occurred, even in the light, although 3-PGA-induced alkalization could be seen. In the presence of 10 mM NaCl, similar results were obtained in MCpt from *S. italica* (Fig. P1-2c) and *P. maximum* (Fig. P1-2d), except that pyruvate induced slow acidification of the medium.

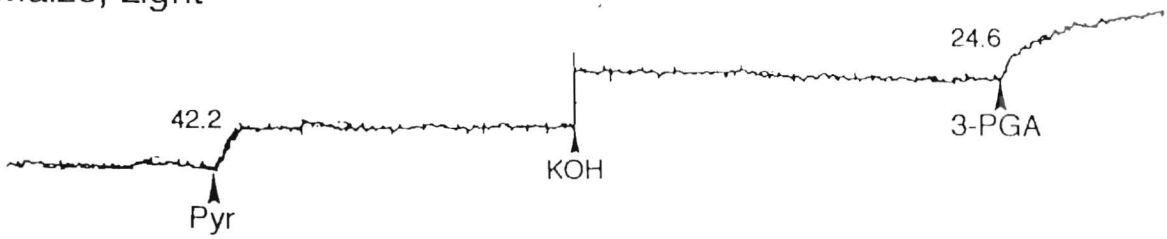
Inhibitors of pyruvate uptake also inhibited pyruvate-induced medium alkalization

Various reagents were tested for the inhibition of pyruvate uptake into MCpt of maize; thiol reagents such as *p*-CMS and Mersalyl are the inhibitors of

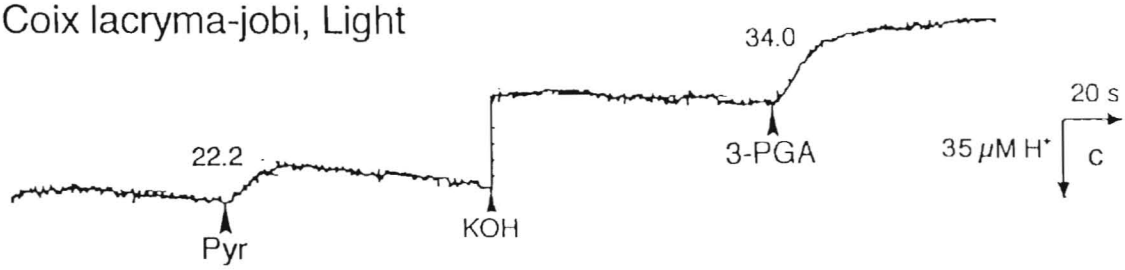
a. Maize, Dark



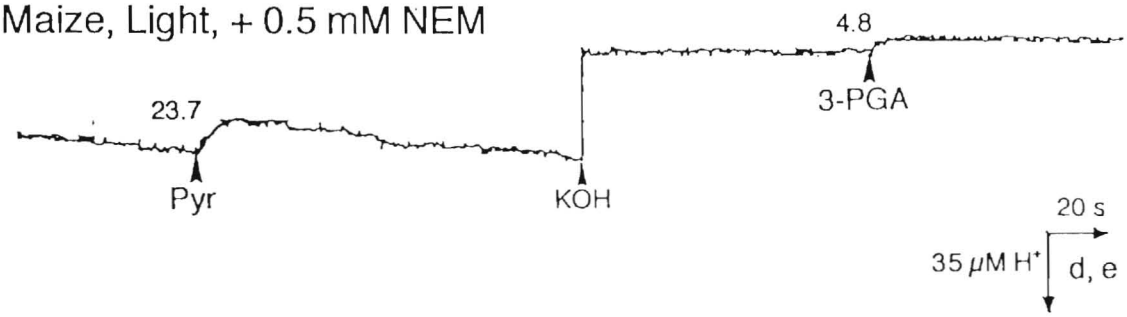
b. Maize, Light



c. Coix lacryma-jobi, Light



d. Maize, Light, + 0.5 mM NEM



e. Maize, Light, + 0.5 mM NBD-Cl

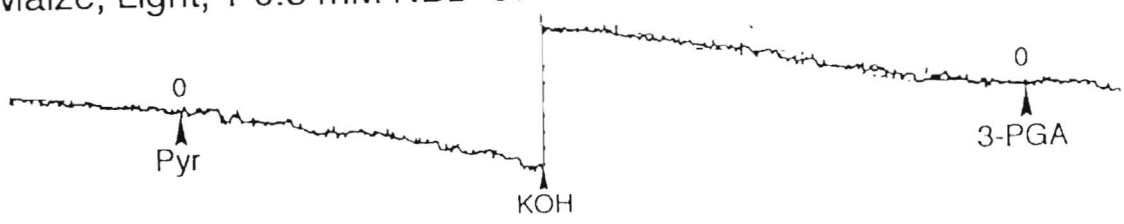


Fig. P1-1. Medium alkalization accompanied with the addition of pyruvate and 3-PGA in mesophyll chloroplast suspension from H^+-type C_4 species, maize (a, b, d and e) and *Coix lacryma-jobi* (c). Chloroplasts were preincubated for about 5 min in the dark, with an inhibitor where indicated (d and e). Then, after 5 min illumination (b to e) or in continuous darkness (a), pyruvate (Pyr in figures, 1.0 mM final concentration) and subsequently 3-PGA (0.5 mM final concentration) was added. Numbers on the pH traces indicate H^+ consumption rates in the medium calculated from the initial slopes (μmol / mg Chl / h). pH of the medium was adjusted to 7.0 before the addition of substrate.

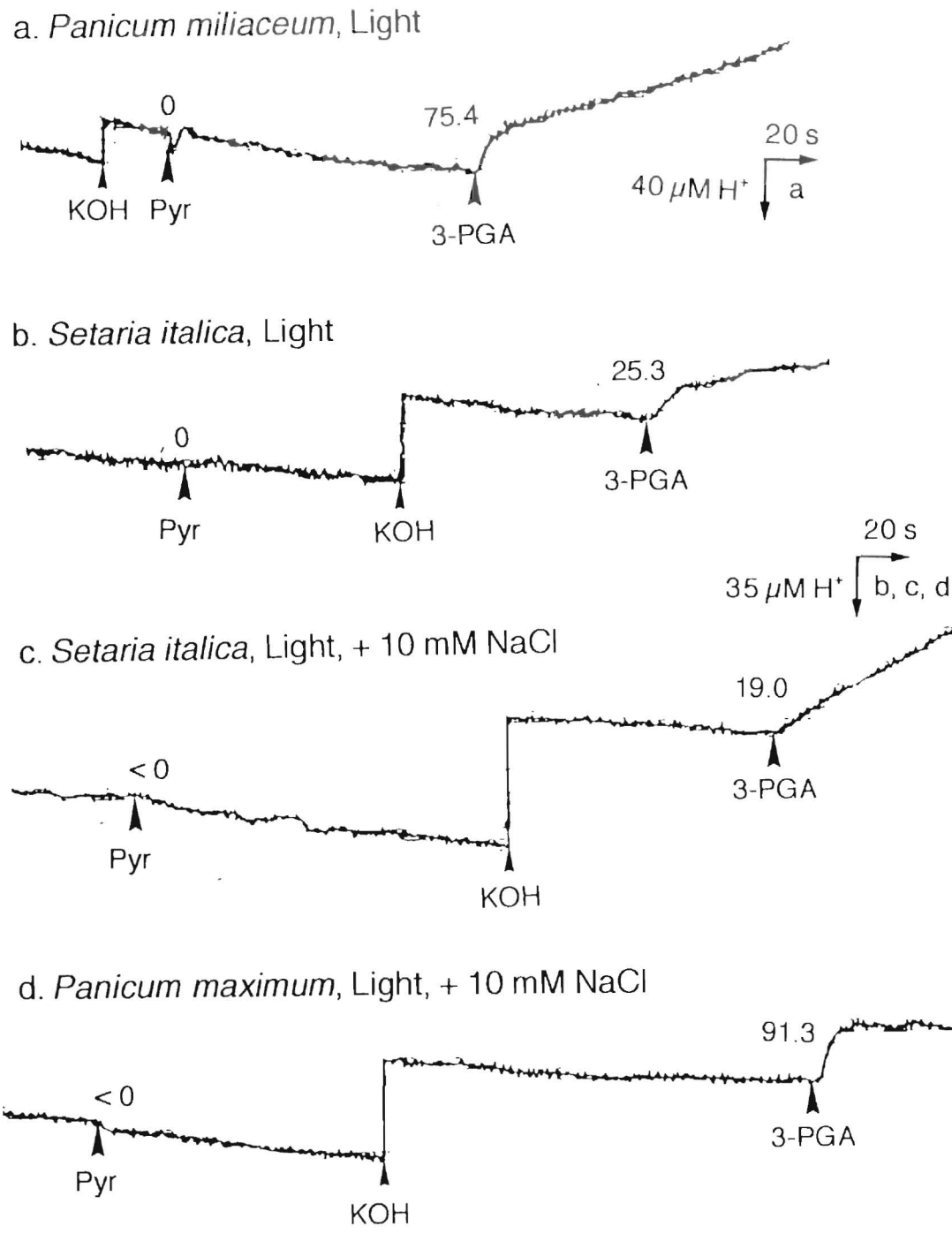


Fig. P1-2. Medium pH changes accompanied with the addition of pyruvate and 3-PGA in mesophyll chloroplast suspension from Na^+-type C_4 species, *Panicum miliaceum* (a), *Setaria italica* (b and c), and *P. maximum* (d)

Refer to the legend of Figure P1-1 for details.

pyruvate uptake into MCpt from *P. miliaceum* (Ohnishi and Kanai 1987a), another thiol reagent NEM (Flügge and Heldt 1986) and a lysine modifying reagent DIDS are known as the inhibitors of chloroplast Pi translocator (Rumpho and Edwards 1985, Gross et al. 1990), while α-CHCA is a pyruvate analogue and a protein modifying inhibitor of pyruvate transport into mitochondria (Halestrap 1975). The rate of pyruvate uptake into maize MCpt was measured at pH 7.0 in the light after incubation with these substances in the dark. Among the five reagents, NEM inhibited pyruvate uptake much more than the others, while *p*-CMS was ineffective (Table P1-1). Thus, NEM and NBD-Cl were selected for further experiments, since the latter was also shown to be a strong inhibitor of pyruvate uptake in MCpt of sorghum, another H^+-type species (Ohnishi et al. 1992).

As shown in Figure P1-1d and -1e, the rate of pyruvate-induced alkalization in the light was reduced to 56% and 0% in the presence of 0.5 mM NEM and NBD-Cl, respectively. 3-PGA-induced alkalization was also inhibited by both inhibitors.

Figure P1-3 shows the concentration dependency of the inhibition of pyruvate uptake by NEM and NBD-Cl. The concentration giving 50% inhibition was about 0.25 mM for NEM and about 0.025 mM for NBD-Cl.

Comparison of the rates of H^+ uptake and the rates of pyruvate uptake into C_4 mesophyll chloroplasts

Table P1-2 shows comparison of the rates of pyruvate-induced H^+ uptake (A) calculated from the data shown in Figures P1-1 and -2 and the rates of [^{14}C]pyruvate uptake (B) measured at the same condition (at 4 to 5°C and 1.0 mM pyruvate) using the aliquots of the same MCpt preparation. In H^+-type species, H^+ uptake showed a quantitative correlation with pyruvate uptake with

Table P1-1. Effects of various reagents on the initial rate of [^{14}C]pyruvate uptake into maize mesophyll chloroplasts in the light

Condition		Rate of Pyruvate Uptake (μmol / mg Chl / h)
Dark		0
Light		18.2±0.1(100)
	+ 0.1 mM NEM	12.5±0.5 (69)
	+ 0.5 mM NEM	9.4±0.3 (52)
	+ 0.1 mM α-CHCA	14.3±0.8 (79)
	+ 0.5 mM α-CHCA	11.6±0.1 (64)
	+ 0.1 mM DIDS	14.8±0.2 (81)
	+ 0.5 mM DIDS	13.5±0.2 (74)
	+ 0.1 mM Mersalyl	14.1±0.4 (77)
	+ 0.5 mM Mersalyl	11.1±0.4 (61)
	+ 0.1 mM *p*-CMS	16.9±0.5 (93)
	+ 0.5 mM *p*-CMS	17.9±0.3 (98)

Chloroplasts were preincubated with various substances for 5 min in the dark. Then, after 5 min illumination, [^{14}C]pyruvate (0.2 mM) uptake for 1 s was measured. The numbers in parentheses indicate relative values.

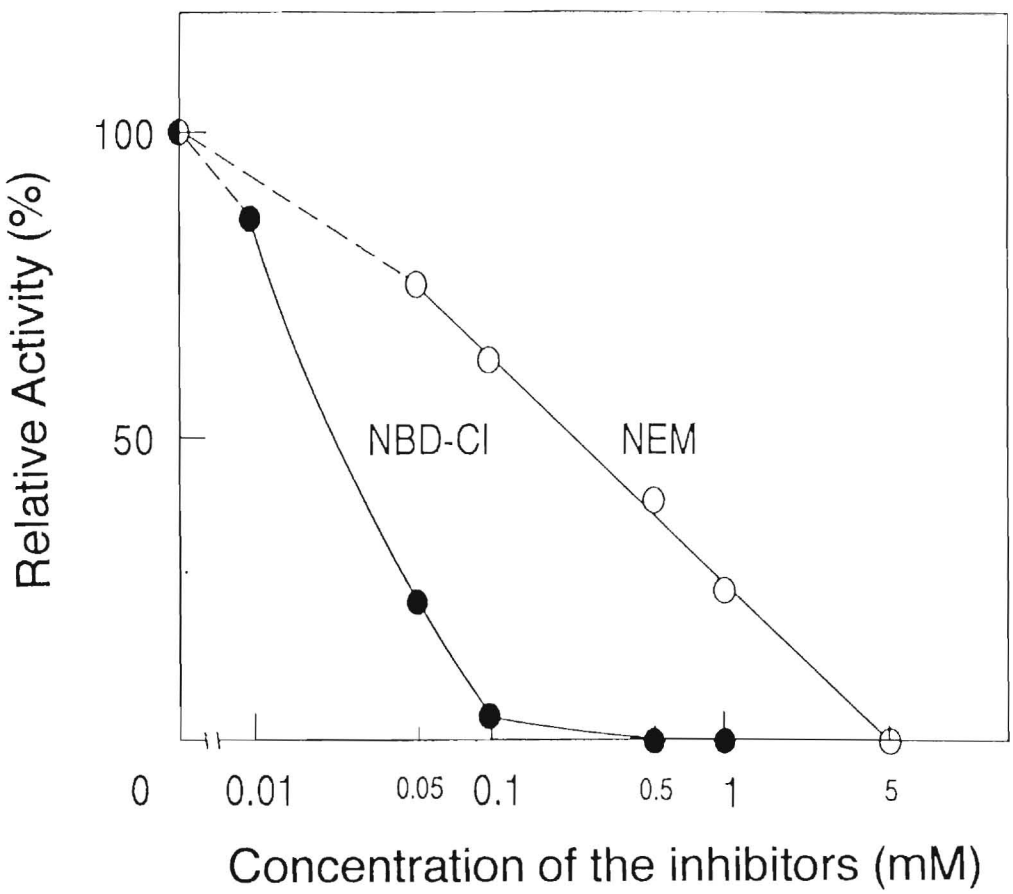


Fig. P1-3. Effects of NEM and NBD-Cl on [^{14}C]pyruvate uptake into maize mesophyll chloroplasts In the light

Chloroplasts were preincubated with various concentrations of the inhibitors (NEM, o; NBD-Cl, •) for 5 min in the dark. Then, after 5 min illumination, [^{14}C]pyruvate uptake in 1 s was measured. The control activity was 27.7 μmol / mg Chl / h.

Table P1-2. Rates of H^+ and pyruvate uptake into mesophyll chloroplasts from various C_4 plants

Species	Condition	Rate of H^+ Uptake (A)	Rate of Pyruvate Uptake (B)	A / B
H^+-type				
Maize	Dark	0	0	-
	Light	42.4	47.3	0.90
	+ 0.5 mM NEM	23.7	23.6	1.00
	+ 5 mM NEM	0	0	-
	+ 0.05 mM NBD-Cl	13.7	14.2	0.96
	+ 0.5 mM NBD-Cl	0	0	-
Coix lacryma-jobi	Dark	2.8	0	-
	Light	22.2	17.9	1.08[a]
Na^+-type				
Panicum miliaceum	Light	0	not determined	-
Setaria italica	Light	0	25.3	-
	+ 10 mM NaCl	< 0	31.8	-
Panicum maximum	Light + 10 mM NaCl	< 0	19.0	-

Rates of H^+ uptake (A, μmol H^+ / mg Chl / h) were obtained from the same sets of experiments as shown in Figures P1-1 and -2. Rates of pyruvate uptake for 1 s (B, μmol / mg Chl / h) were measured using chloroplasts from the same mesophyll protoplast preparations. [a] The dark value of (A) was subtracted from the light value.

the ratio of A to B being about 1. In Na^+-type species, on the other hand, no H^+ uptake was observed at all on pyruvate addition in spite of high rates of pyruvate uptake.

pH change in the external medium induced by the addition of PEP in mesophyll chloroplasts from maize and *P. miliaceum*

Figure P1-4 shows a result tracing the change in external pH of maize MCpt suspension in the light. As shown in Figure P1-1b, when 1 mM pyruvate was added to the chloroplast suspension at pH 7.0, pH of the medium increased within 10 s and kept the level. Subsequent addition of 0.5 mM PEP induced remarkable alkalization and slow return to the original pH. Then, the addition of 0.5 mM 3-PGA confirmed the rapid alkalization as seen in C_3 chloroplasts (Fliege et al. 1978). This line of experiment in maize was compared with that in *P. miliaceum*. Table P1-3 summarizes the initial rates of H^+ uptake accompanied with the addition of pyruvate, PEP and 3-PGA in sequence, as measured in Figure P1-4. The H^+ uptake on addition of PEP or 3-PGA were observed in both dark and light, and in both maize and *P. miliaceum*. In maize MCpt, the rates of H^+ uptake on 3-PGA addition in the light were about half of those in the dark. In contrast, in *P. miliaceum*, the rates in the light were about twice in the dark. Although the significance of these results is obscure because of pyruvate and PEP additions prior to 3-PGA in these experiments, the rates of 3-PGA-induced H^+ uptake are comparable to those reported in C_3 chloroplasts (Fliege et al. 1978).

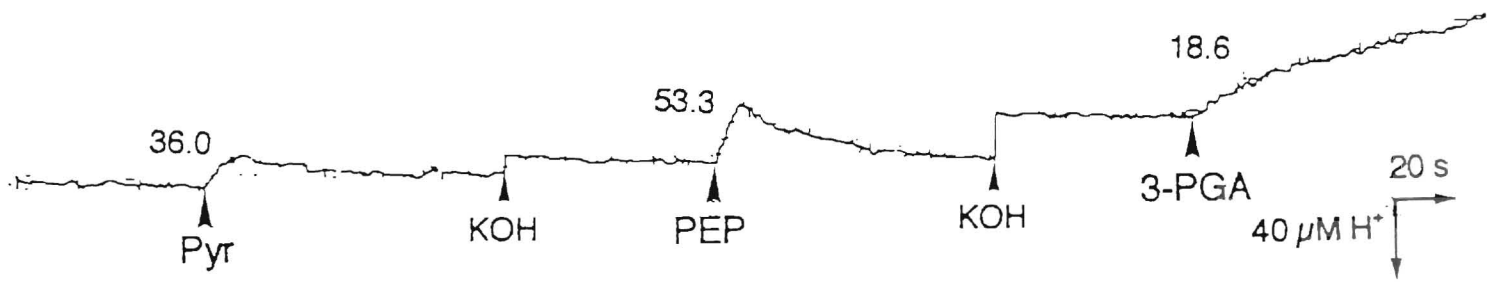


Fig. P1-4. Alkalization of medium by the addition of pyruvate, PEP and 3-PGA to mesophyll chloroplast suspension from maize in the light.

Pyruvate (1.0 mM final concentration), PEP (0.5 mM final concentration) and 3-PGA (0.5 mM final concentration) were added in sequence. Refer to the legend of Figure P1-1 for detail.

Table P1-3. Rates of H^+ consumption in the external medium accompanied with the addition of pyruvate, PEP and 3-PGA in sequence

		Rate of H^+ Uptake (μmol / mg Chl / h)		
		+ Pyr	+ PEP	+ 3-PGA
Maize				
Dark	Pyr → PEP → 3-PGA	0.0	70.0	31.2
Light	Pyr → PEP → 3-PGA	36.0	53.3	18.6
	PEP → Pyr → 3-PGA	11.7	64.1	19.6
Panicum miliaceum				
Dark	Pyr → PEP → 3-PGA	0.0	144.0	19.4
Light	Pyr → PEP → 3-PGA	0.0	80.0	53.2

Experimental details were as in Fig. P1-4.

Effect of PEP on pyruvate-induced alkalization in the medium and acidification in stroma of maize mesophyll chloroplasts

H^+ uptake on addition of pyruvate was observed only in C_4 MCpt of H^+-type in the light, and H^+/pyruvate cotransport ratio of one. It should be noted that the rate of H^+ uptake by pyruvate addition (36.0 μmol / mg Chl / h) was decreased to about one third (11.7 μmol / mg Chl / h) when PEP was added prior to pyruvate (Table P1-3).

When pyruvate (1.0 mM at final concentration) was added to maize MCpt, pH in the stroma decreased by about 0.3 units within 10 s and thereafter almost recoverd after 5 minutes (● in Fig. P1-5). This initial rapid change corresponded well to the pH change in the medium (Fig. P1-1b and -4). This stromal acidification was reduced to 75% in the presence of 1.0 mM PEP (▲ in Fig. P1-5). However, the initial rate of [^{14}C]pyruvate uptake into maize MCpt in the light, measured by the double layer system, showed no significant change in the presence and absence of PEP (Table P1-4). The results indicate that pyruvate uptake itself is not affected by PEP.

[^{14}C]PEP uptake into maize mesophyll chloroplasts

It has been known that the Pi translocator of C_4 MCpt catalyses a exchange of Pi, triose phosphate, 3-PGA, 2-PGA and PEP. These were demonstrated by a competitive inhibition against [^{32}P]Pi transport (Huber and Edwards 1977b, Gross et al. 1990). In this method, however, it is unclear whether the latter four compounds are transported via a common or separate translocator protein(s). To test this possibility, the initial rate of [^{14}C]PEP uptake into maize MCpt was compared with the presence of Pi, 3-PGA or 2-PGA (Fig. P1-6). [^{14}C]PEP (0.04 to 0.2 mM) plus unlabeled substrates (Pi, 3-PGA or 2-PGA) were contained in the uptake layer of the double layer system. All of

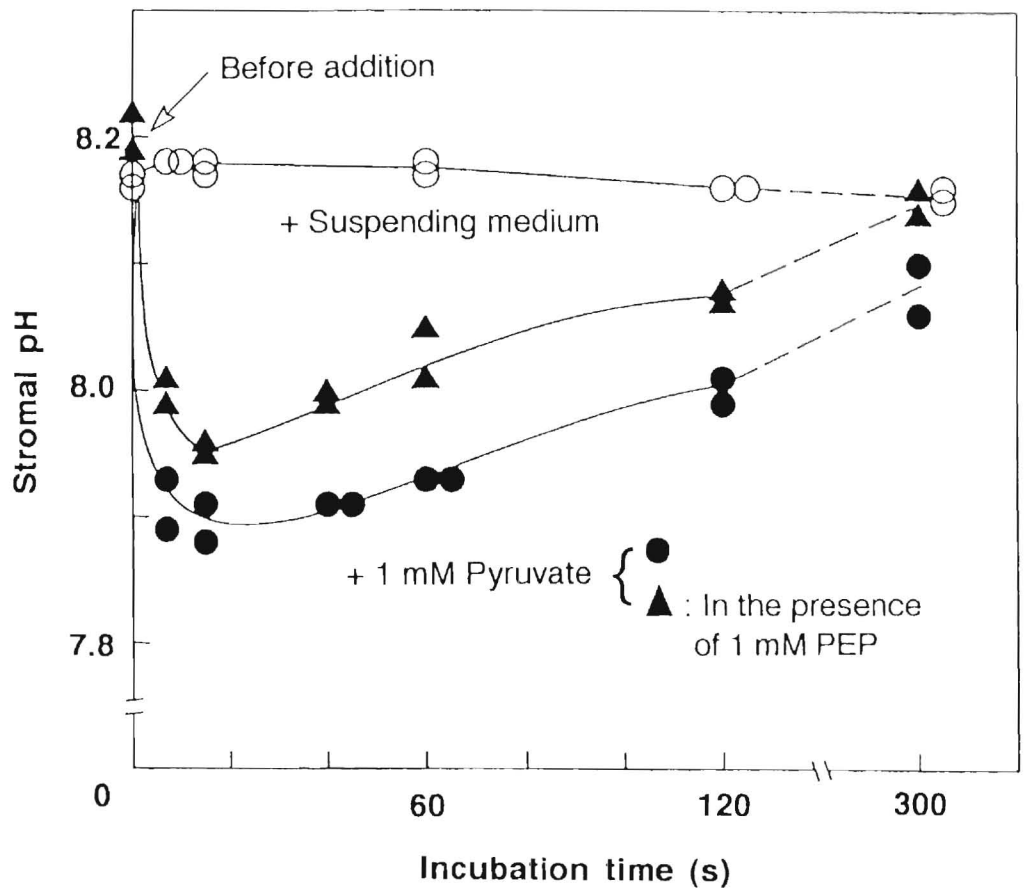


Fig. P1-5. Effect of PEP on pyruvate-induced stromal acidification in maize mesophyll chloroplasts in the light

Chloroplasts were preincubated with [^{14}C]DMO and [^{3}H]sorbitol for 5 min in the light, and with 1 mM PEP where indicated (▲). Then, pyruvate (● and ▲, 1.0 mM final concentration) or the same volume of a suspending medium (o) was added at zero time.

Table P1-4. Effect of PEP on the initial rate of [^{14}C]pyruvate uptake into maize mesophyll chloroplasts

PEP (0.5 mM) was added to both the chloroplast and the uptake layers of the double layer system.

The reaction time was 2 s.

		Rate of pyr uptake (μmol / mg Chl / h)
Dark	Control	0.5±0.1
Light	Control	34.2±0.7
	+ 0.5 mM PEP	33.9±0.4

$[pyr]_{ex}$ = 1.0 mM.

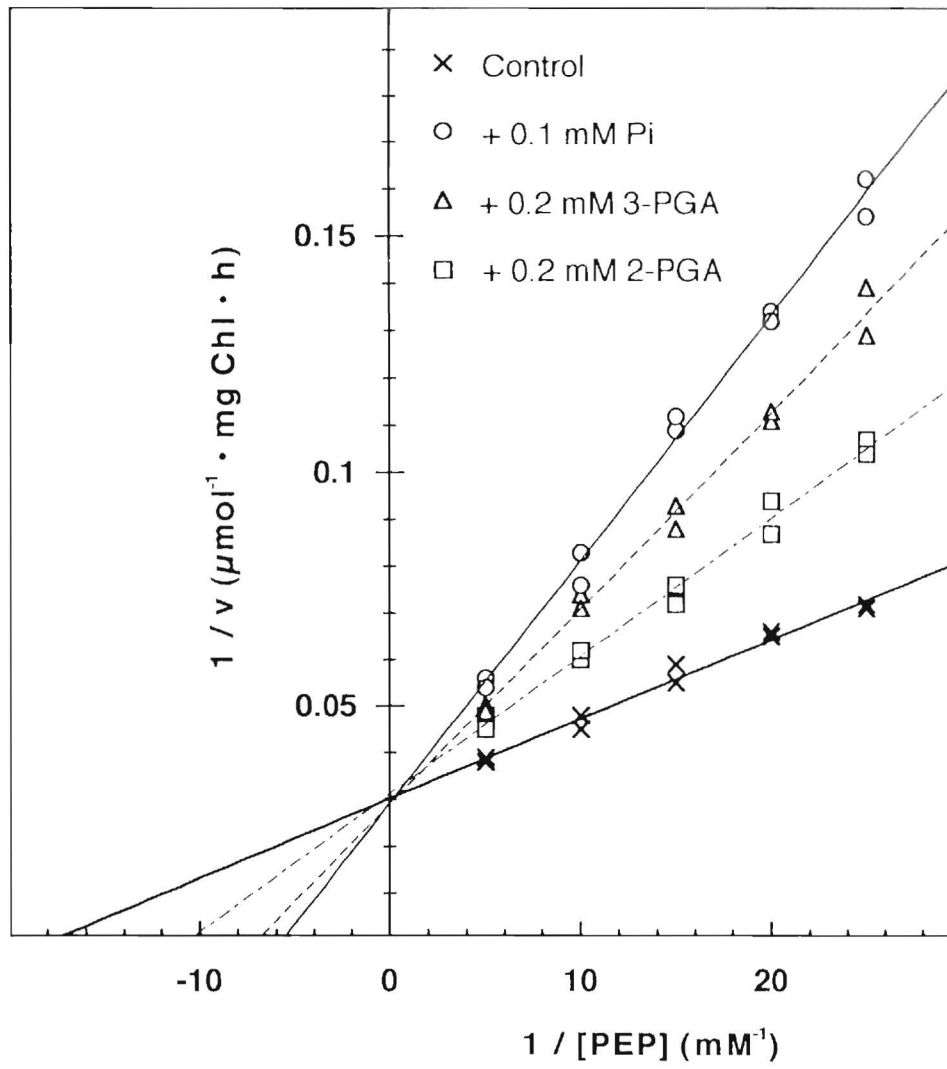


Fig. P1-6. [^{14}C]PEP uptake into maize mesophyll chloroplasts

Double reciprocal plots of initial rate of PEP uptake. Pi, 3-PGA and 2-PGA were added to the uptake layer of the double layer system. The reaction time was 2 s.

the unlabeled substrates competitively inhibited the rate of [^{14}C]PEP uptake. Km value for PEP (0.059 mM) was similar to Ki for Pi (0.051 mM), but a little lower than Ki for 3-PGA or 2-PGA (0.15 mM and 0.27 mM, respectively).

Changes in the rate of pyruvate uptake, stromal pH, ΔpH across the envelope and ΔpH (Light minus Dark) in stroma of maize mesophyll chloroplasts in the medium of various pH

Figure P1-7a shows the effects of external pH on the rate of pyruvate uptake into maize MCpt. Pyruvate uptake was induced by illumination, but not by Na^+-jump both in the dark and in the light. These results indicate that Na^+ is not transported together with pyruvate in MCpt of H^+-type C_4 species, consistent with the comparative study of the cation specificity in many C_4 species (Aoki et al. 1992, Table I-1). In the range of external pH from 7.0 to 8.6, light-dependent activity was highest at pH 7.0 and declined when external pH was raised, and little activity was detected at pH 8.6 (o and Δ in Fig. P1-7a). The pH dependency of light-dependent pyruvate uptake was quite similar to that of ΔpH between the stroma and the external medium in the light (o and Δ in Fig. P1-7c), as well as to that of pH difference in stroma between in the light and the dark (x and + in Fig. P1-7c).

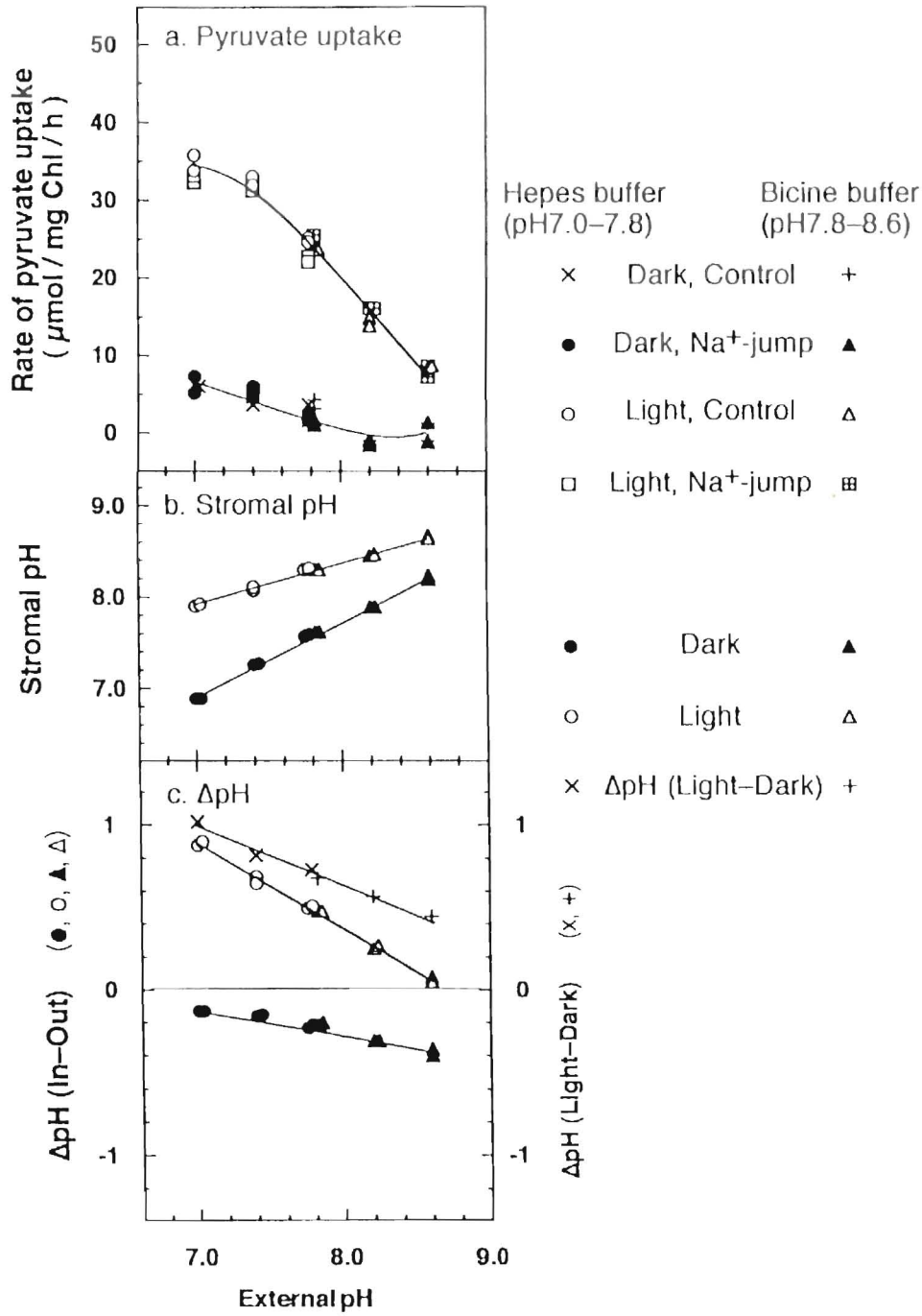


Fig. P1-7. External pH dependency of pyruvate uptake, stromal pH, ΔpH (In minus Out) and ΔpH (Light minus Dark) in mesophyll chloroplasts of maize

After centrifugation, MCpt was resuspended in the medium of verious pH. Hepes buffer was used at pH 7.0, 7.4 and 7.8, and Bicine buffer at pH 7.8, 8.2 and 8.6. The rates of pyruvate uptake in four conditions (a) and stromal pH (b) were measured. ΔpH (In minus Out) and ΔpH (Light minus Dark) were calculated from the measurements of stromal pH. The time of pyruvate uptake ($[pyr]_{ex}$ = 1.0 mM) was 2 s.

Discussion

H^+ transport accompanied with 3-PGA transport in C_4 mesophyll chloroplasts; Assessment of the pH measuring system

The Pi translocator protein is present in the mesophyll chloroplast envelope of C_3 and C_4 plants and transports Pi, 3-PGA and triose phosphate in a manner of strict counter exchange (Flügge and Heldt 1991). Fliege et al. (1978) reported that when 3-PGA was added to C_3 chloroplasts, medium pH increased in time well corresponding to that of 3-PGA uptake. This can be explained by assuming that the Pi translocator mediates the strict exchange of divalent anions and that 3-PGA transport is accompanied by indirect H^+ transport, since 3-PGA is a trivalent anion in a physiological pH range (Fliege et al. 1978). As shown in Figures P1-1 and -2, 3-PGA-induced alkalization in the medium was also observed in MCpt of all the C_4 species tested. The initial rate of this alkalization was 24.6 to 91.3 μmol H^+ / mg Chl / h and the rapid phase of H^+ decrease corresponded to 65 to 175 nmol H^+ / mg Chl. These values are comparable with the results in Fliege et al. (1978): 20 μmol H^+ / mg Chl / h and 140 nmol H^+ / mg Chl, respectively. These results indicate that our pH measuring system is sensitive enough to detect H^+ changes accompanied with metabolite transport across the chloroplast envelope. Furthermore, this medium alkalization was inhibited by NEM, the reported inhibitor of the Pi translocator (Flügge and Heldt 1986) and also by NBD-Cl, a potent inhibitor of pyruvate transport (Ohnishi et al. 1992) (Fig. P1-1d and -1e, and Table P1-2).

The effects of various protein modifying reagents on the rate of pyruvate uptake into C_4 mesophyll chloroplasts

Pyruvate uptake into MCpt from maize, a H^+-type C_4 species, at pH 7.0 in

the light, was inhibited by NEM but not by *p*-CMS (Table P1-1). In contrast, [^{14}C]pyruvate uptake into MCpt from *P. miliaceum*, a Na^+-type C_4 species, at pH 7.8 in the light, was inhibited to about 20% by 0.1 mM *p*-CMS, but not by 1 mM NEM (Ohnishi and Kanai 1987a). The results may reflect some differences between Na^+- and H^+-type C_4 species in the manner of pyruvate uptake, possibly due to a different carrier protein(s) in transport of Na^+ and H^+.

In this study, NEM and NBD-Cl were used as inhibitors of pyruvate uptake in MCpt of H^+-type. However, it should be noted that both reagents also apparently inhibited the Pi translocator as seen in Fig. P1-1d, -1e and Table P1-2.

H^+ uptake accompanying pyruvate uptake into mesophyll chloroplasts from H^+-type C_4 species

In H^+-type C_4 species, pyruvate-induced alkalization in the medium (Fig. P1-1 and Table P1-2) and acidification of chloroplast stroma (Fig. P1-5) were observed in the light. The former change was completely inhibited in the presence of 5 mM NEM and 0.5 mM NBD-Cl (Fig. P1-1 and Table P1-2) which also inhibited [^{14}C]pyruvate uptake completely (Fig. P1-3 and Table P1-2). In Na^+-type C_4 species, pyruvate-induced alkalization in the medium was not observed (Fig. P1-2). These results suggest that H^+/pyruvate cotransport occurs only in the MCpt of H^+-type C_4 species in the light. Although pyruvate (pK_a = 2.5) is present almost exclusively as a monovalent anion in a physiological pH range, pyruvate seems to be electroneutrally cotransported with H^+ in the light, because the ratio of pyruvate and H^+ transport is about 1 (Table P1-2). This conclusion is in contrast with that of Huber and Edwards (1977), who suggested an electrogenic pyruvate uptake in MCpt of *Digitaria sanguinalis* in the dark.

The rate of H^+ uptake obtained with our system (20 to 50 μmol / mg Chl / h) seem to be large enough to explain the net photosynthetic rate, considering that the experimental temperature at 4°C.

PEP transport via the Pi translocator in C_4 mesophyll chloroplasts

As PEP is transported via the Pi translocator in C_4 MCpt (Huber and Edwards 1977b, Gross et al. 1990), alkalization of the medium due to PEP addition (Fig. P1-4 and Table P1-3) indicates that PEP is transported together with H^+ as a divalent anion into C_4 MCpt, similar to 3-PGA transport, as mentioned above. The initial rates of H^+ uptake on PEP addition were much higher than those on 3-PGA addition (Table P1-3). Therefore, H^+ flux accompanied with PEP transport across the envelope of C_4 MCpt is physiologically significant.

Kinetical analysis of [^{14}C]PEP transport in maize MCpt (Fig. P1-6) indicate that PEP and Pi, 3-PGA or 2-PGA could be transported by the same translocator protein. Gross et al. (1990) reported in maize MCpt that, using [^{32}P]Pi as a substrate, Km for Pi was 0.045 mM, and Ki for PEP, 3-PGA and 2-PGA were 0.086, 0.053 and 0.073 mM, respectively. In comparison with these values, my determination of Km for PEP (0.059 mM) and Ki for Pi (0.051 mM) were similar but Ki for 3-PGA (0.15 mM) and 2-PGA (0.27 mM) were a little higher. The results may indicate that, as an exchange substrate for PEP transport in maize MCpt, Pi is more favorable than 3-PGA and 2-PGA.

Effect of PEP on pyruvate uptake into C_4 mesophyll chloroplasts

When PEP was preincubated with maize MCpt, both medium alkalization (Table P1-3) and stromal acidification (Fig. P1-5) due to pyruvate addition were decreased, while the rate of pyruvate uptake itself was not changed

(Table P1-4). The result indicates that PEP supports only for H^+ transport without affecting H^+/pyruvate cotransport into maize MCpt. Thus, H^+ influx due to H^+/pyruvate cotransport is compensated by H^+ efflux accompanied with PEP export via the Pi translocator.

Stromal alkalization, H^+ gradient across the envelope and active pyruvate uptake in maize mesophyll chloroplasts

It has been suggested that active pyruvate uptake into MCpt of H^+-type C_4 species is driven by the H^+ gradient formed across the envelope in the light (Ohnishi and Kanai 1990). Actually, stromal pH in maize MCpt was shifted from about 7 to 8 by illumination at neutral pH of the medium (Fig. P1-7b and -7c). External pH dependency of light-dependent pyruvate uptake was changed in parallel with that of stromal alkalization and that of ΔpH across the envelope, both of which occurred by illumination. Therefore, H^+ gradient formed across the envelope by illumination is the driving force of active pyruvate uptake into MCpt of H^+-type C_4 species.

Conclusion

Figure P1-8 illustrates the event occurring in the mesophyll chloroplast of H^+-type C_4 species such as maize *in vivo* in the light. The cytosolic pH is supposed to be neutral. On illumination of MCpt, the stroma alkalizes because of H^+ uptake into the thylakoid lumen by photosynthetic electron transport system in the thylakoid. Thus, H^+ gradient formed between the cytosol and the stroma is used for the active transport of pyruvate; pyruvate and H^+ are cotransported in a ratio of one. The pyruvate is converted to PEP by pyruvate,Pi dikinase, which is located in the MCpt, and then the H^+ cotransported with pyruvate will be released together with the PEP via Pi translocator. Thus, the H^+ gradient between stroma and cytosol is maintained for continuous uptake of pyruvate into MCpt during photosynthesis.

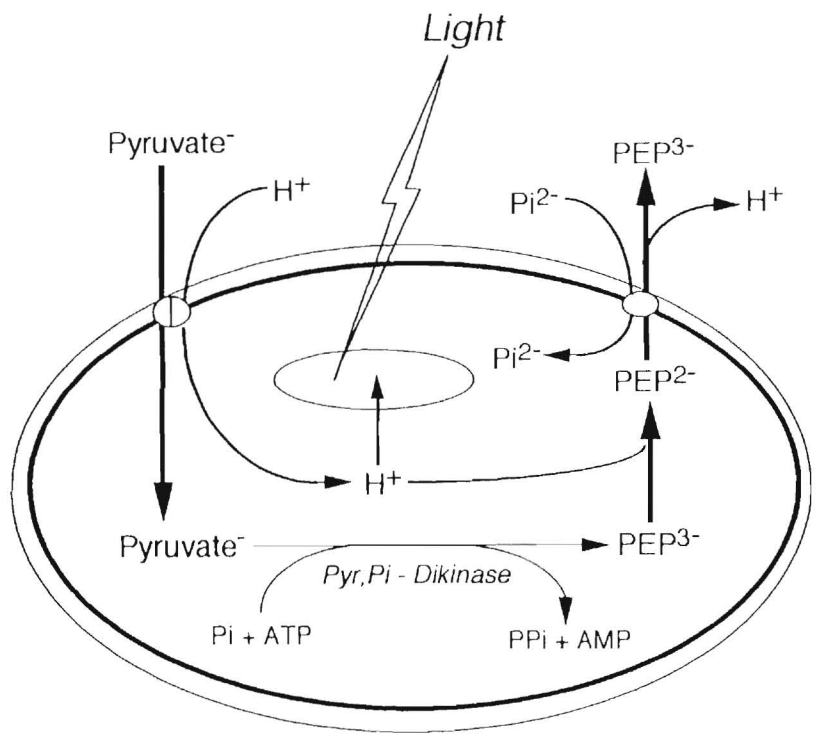


Fig. P1-8. A scheme of H^+ mobilization accompanied with metabolite transports in the light in mesophyll chloroplasts of maize, a H^+-type C_4 species

Part 2

Active transport of pyruvate in the light and a possible role of Na^+ gradient formation across the envelope in mesophyll chloroplasts of *Panicum miliaceum* L., a Na^+-type C_4 species

As to the mechanism of light-dependent active pyruvate uptake into MCpt of Na^+-type C_4 species, it has been reported that Na^+/pyruvate cotransport system in the ratio of one is operating (Ohnishi et al. 1990). Although it has been thought that the driving force for Na^+/pyruvate cotransport is Na^+ gradient formed across the envelope by illumination, there is no evidence for the formation of Na^+ gradient across the envelope in the light, except a small sign of Na^+ release induced by illumination of MCpt of *P. miliaceum* (Ohnishi et al. 1990).

Results

Effects of external pH on the rates of pyruvate uptake into mesophyll chloroplasts of *P. miliaceum*

Figure P2-1a shows a external pH dependency of the rates of pyruvate uptake into MCpt of *P. miliaceum*, measured as described in Figure P1-7a. Pyruvate uptake induced by Na^+-jump in the dark (B in Fig. P2-1a) was independent of external pH from 7.0 to 8.6. The activity of pyruvate uptake in the light (C) was almost the same as that in the dark (A) at pH 7.0 and greatly increased in alkaline pH from 7.4 to 8.6, whereas in maize, the activity in the light was maximum at pH 7.0 and reduced to nearly zero above pH 8.0 (Fig. P1-7a). The light-dependent activities (A in Fig. P2-1a) were further enhanced

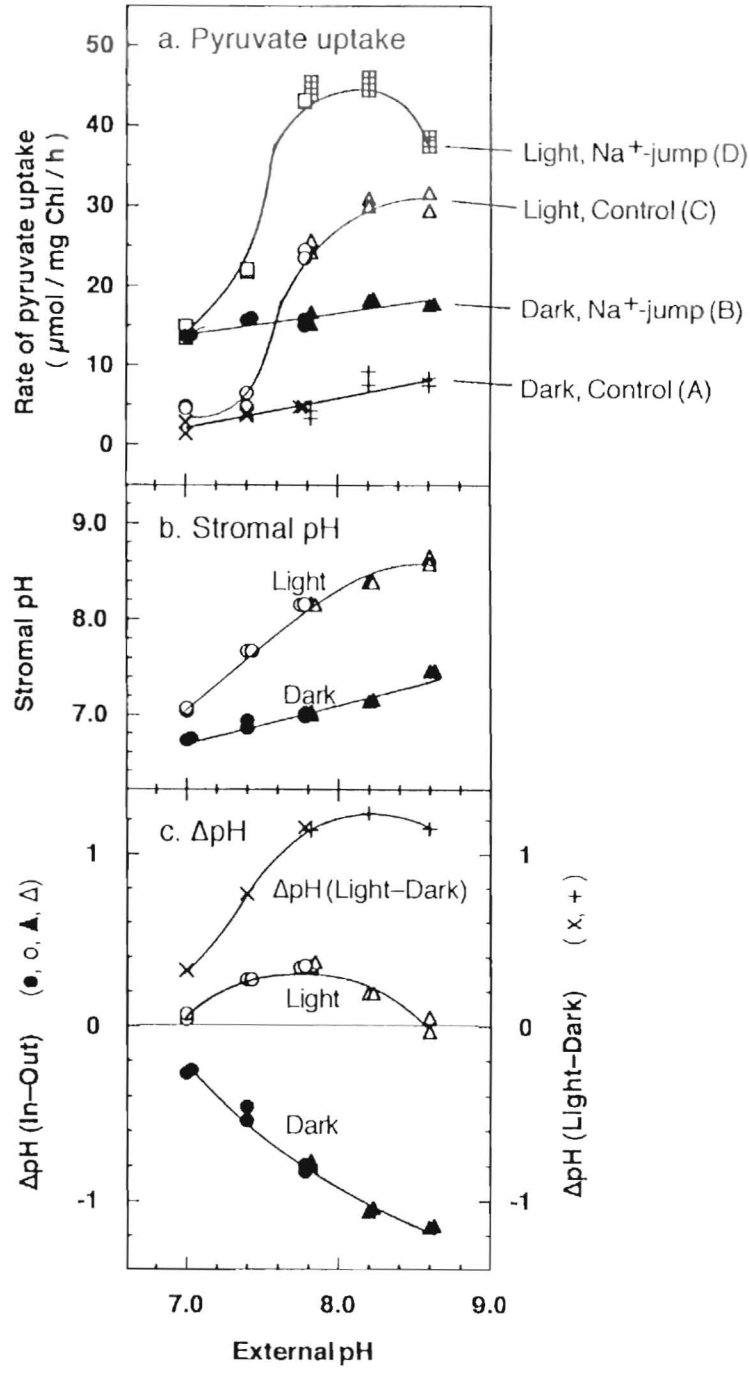


Fig. P2-1. pH dependency of pyruvate uptake, stromal pH, ΔpH (In minus Out) and ΔpH (Light minus Dark) in mesophyll chloroplasts of *P. miliaceum*

Refer to the text and the legend of Figure P1-7 for detail.

by concomitant Na^+-jump in all pH range (D). Essentially the same results were obtained in five independent experiments.

The relationships between light-dependent pyruvate uptake in mesophyll chloroplasts of *P. miliaceum* and (i) stromal pH in the light, (ii) ΔpH formed across the envelope by illumination, or (iii) the difference in stromal pH between the light and the dark

In the same way as in MCpt of maize (Fig. P1-7), external pH dependency of pyruvate uptake activities in MCpt of *P. miliaceum* were compared with that of the ΔpH formed across the envelope by illumination and the difference in stromal pH between the light and the dark (Fig. P2-1b and c).

Compared with maize, one of the most distinguishable points in MCpt of *P. miliaceum* was stromal pH in the dark; it was much lower than the external pH (● and ▲ in Fig. P2-1b). These results may suggest that MCpt of *P. miliaceum* retain H^+ in the dark due to low permeability of the envelope against H^+. Thus, the ΔpH between stroma and medium reached to lower than −1, when MCpt are suspended in the medium at alkaline pH.

The external pH dependency of light-dependent pyruvate uptake (C in Fig. P2-1a) was roughly identical with that of the difference in stromal pH between the light and the dark (x and + in Fig. P2-1c), but different from that of ΔpH formed across the envelope in the light (o and Δ in Fig. P2-1c). Results obtained from five independent experiments were summarized in Fig. P2-2. High light-dependent activities were achieved when the light-and-dark difference in stromal pH was reached over 0.8 units (Fig. P2-2, right panel). Similar result was obtained in relation to stromal pH, while the uptake activity caused by Na^+-jump is independent of stromal pH (Fig. P2-3), as well as external pH (Fig. P2-1a).

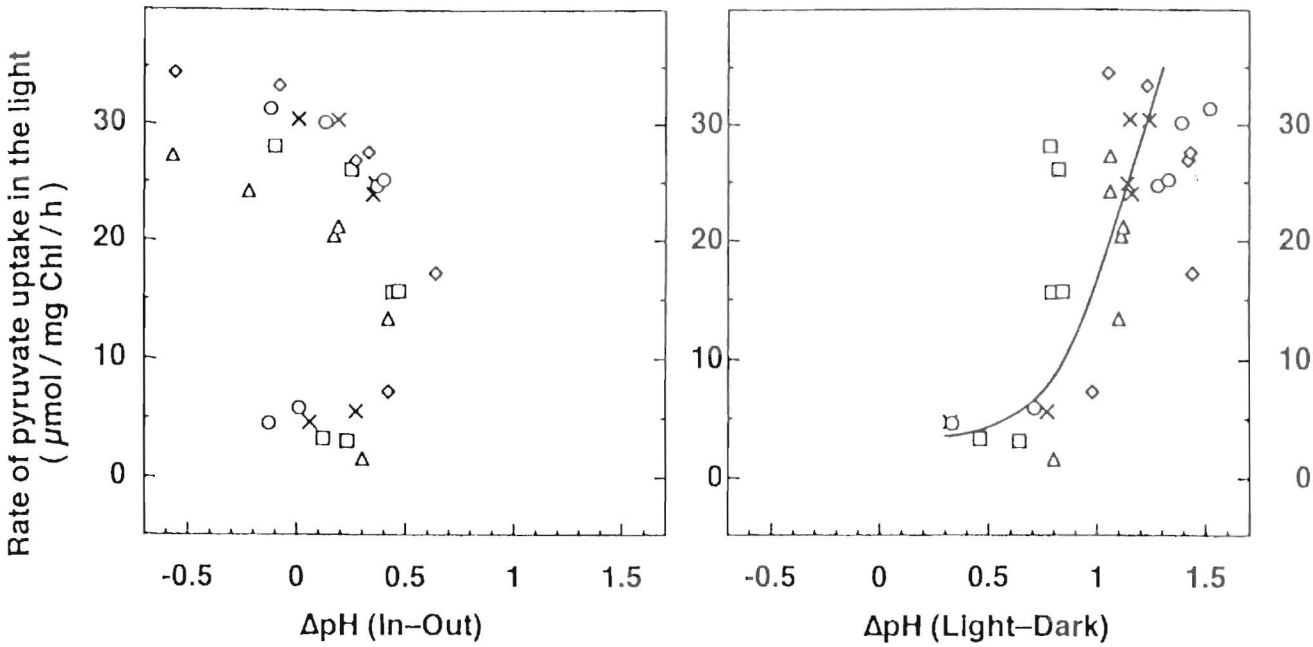


Fig. P2-2. The relationship between light-dependent pyruvate uptake and ΔpH (In minus Out) or ΔpH (Light minus Dark) in mesophyll chloroplasts of *P. miliaceum*

Data from Fig. P2-1 and the other four independent experiments.

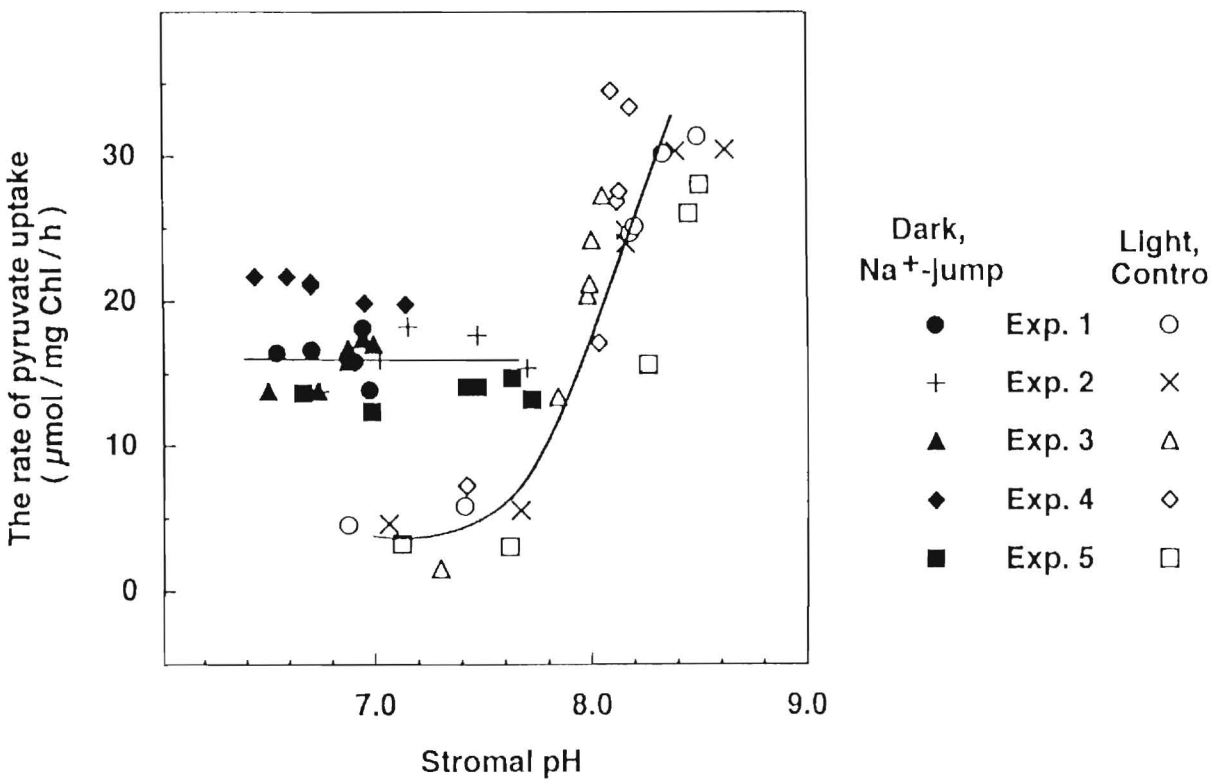


Fig. P2-3. The relationship between stromal pH and pyruvate uptake induced by illumination or Na^+-jump in the dark in mesophyll chloroplasts of *P. miliaceum*

Data from Fig. P2-1 and the other four independent experiments.

A synergistic effect of illumination and Na^+-jump on pyruvate uptake into mesophyll chloroplasts of *P.miliaceum* at alkaline pH

Figure P2-4 shows an analysis of the rates of pyruvate uptake, from the data shown in Figure P2-1a. The activity in the dark (A) was subtracted from the others (B, C and D). "Na^+-jump" effect (filled circles; B minus A) was constant in external pH range from 7.0 to 8.6, whereas "Light" effect (open circles; C minus A) became remarkable at alkaline pH range above 7.5. Noteworthy is the fact that "Na^+-jump plus Light" effect (open squares; D minus A) was larger than the sum of "Na^+-jump" effect and "Light" effect (asterisks) in alkaline pH range. This was seen in all five independent experiments. Preliminary results had already been obtained by Ohnishi and Kanai (1987c). These results indicate that illumination and Na^+-jump had a synergistic effect on the rate of pyruvate uptake in the range of external pH from 7.4 to 8.2 (Fig. P2-4).

Effect of H^+-jump on pyruvate uptake into mesophyll chloroplasts of *P. miliaceum*

In the previous studies (Ohnishi and Kanai 1990, Aoki et al. 1992), H^+-jump (lowering external pH from 7.8 to 6.8) in the dark could not induce pyruvate uptake into MCpt from Na^+-type C_4 species. However, in MCpt from *P. miliaceum*, stromal pH in the dark was about 7.0 when external pH was 7.8 (Fig. P2-1b). Thus, lowering external pH from 7.8 to 6.8 would not achieve enough H^+ gradient across the chloroplast envelope in MCpt of *P. miliaceum*. To obtain large H^+ gradient in the dark across the envelope of *P. miliaceum*, the effects of H^+-jump from pH 8.6 to pH 7.6, 6.8 or 6.5 on the rate of pyruvate uptake were checked by the double layer system (Table P2-1). When MCpt were suspended in the medium adjusted at pH 8.6, using Bicine as a buffer, stromal pH in the dark was 7.51 (Table P2-1). Unexpectedly, the "Sorbitol

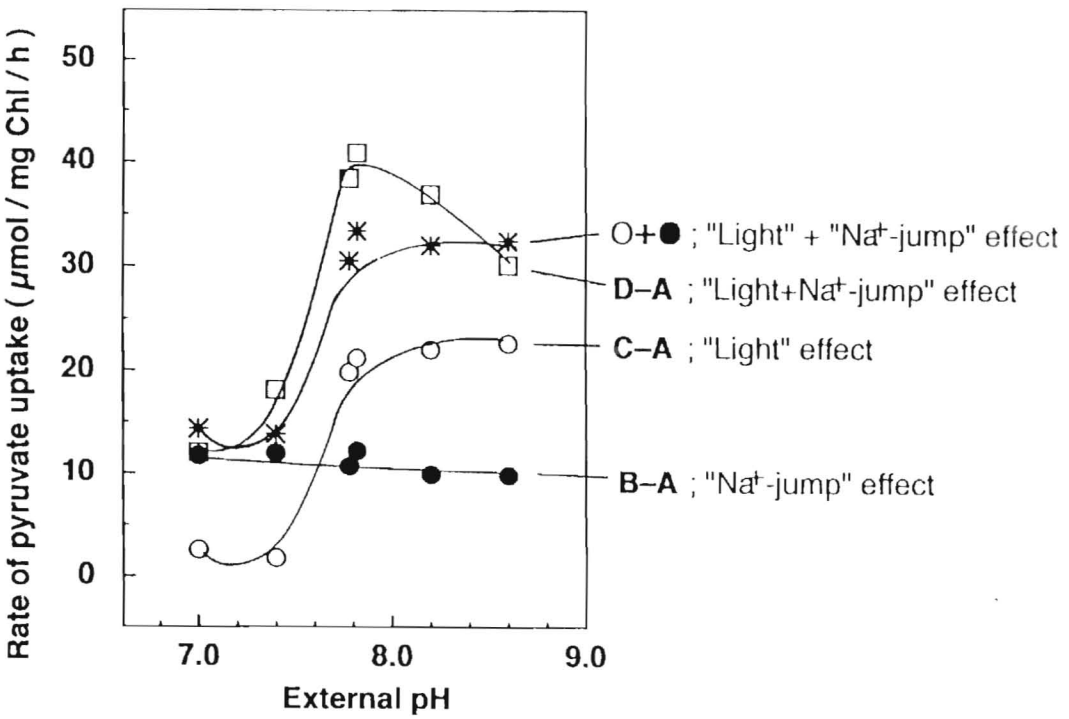


Fig. P2-4. Analysis of pyruvate uptake into mesophyll chloroplasts of *P. miliaceum*

Data from Fig. P2-1. The pyruvate uptake activity in the dark (A) was subtracted from the others (B, C and D).

Table P2-1. Effect of H^+-jump on "Sorbitol Space" in the double layer system

MCpt was suspended in medium adjusted at pH 8.6, using Bicine as a buffer. Then, by the double layer system, the rate of pyruvate uptake was measured. Stromal pH in the dark was pH 7.51±0.03.

Condition	"Space" (μl / mg Chl)		
	[^{3}H]sorbitol	[^{14}C]pyr	Rate*
Light control	36.3	50.1	24.2
Dark control	35.3	38.0	4.4
Dark, Na^+-jump	35.7	43.3	14.4
Dark, H^+-jump, to pH 7.6 (Hepes)	60.6	38.2	< 0
Dark, H^+-jump, to pH 6.8 (Mes)	70.4	38.6	< 0
Dark, H^+-jump, to pH 6.5 (Mes)	73.8	37.9	< 0

* : μmol / mg Chl / h.

Space" in the MCpt precipitate after centrifugation was extraordinarily enlarged by lowering the pH to 7.6, 6.8 or 6.5, using Hepes, Mes or Mes, respectively, as a buffer. Consequently, pyruvate uptake in the dark was apparently inhibited by the H^+-jumps. As these buffers have pKa values at the pH range of the experiment, permeability of the buffers through the envelope will be changed according to the medium pH. To reduce such an effect of buffers, H^+-jump experiments were performed in the medium containing minimum amount of buffer, 5 mM Hepes. H^+-jump was performed by the addition of 0.2 N HCl to MCpt suspension at pH 8.2. No enlargement of "Sorbitol Space" was occurred by this procedure. Table P2-2 shows the effects of light, Na^+-jump and H^+-jump on pyruvate uptake for 10 s into MCpt of *P. miliaceum*, by the single layer system. When pH of the medium was lowered from pH 8.2 to 7.4 (H^+-jump A) in the dark, pH difference between the stroma and the external medium (ΔpH (In–Out)) was –0.3 and pyruvate uptake was not induced (Table P2-2). Lowering external pH from 8.2 to 7.0 or 6.7 (H^+-jump B or C), by which ΔpH (In–Out) were +0.1 or +0.4, respectively, induced some pyruvate uptake in the dark; the rate was lower than the effect of illumination or Na^+-jump. These results may suggest that besides Na^+ gradient, H^+ gradient across the envelope contributes in some extent to pyruvate uptake in alkaline pH range. However, actual H^+ gradient across the envelope could not be formed in H^+-jump B in the dark, because ΔpH (In–Out) was +0.1. No reasonable explanation is possible on this observation, except that stromal pH might be changed by these H^+-jump experiments. Pyruvate uptake into MCpt was also observed when the H^+-jumps were performed in the light (Table P2-2). Some of this pyruvate uptake activity would be caused by H^+ gradient across the envelope (ΔpH (In– Out) > +0.6), since the rate of light-dependent pyruvate uptake was very low in external pH below 7.4 (Fig. P2-1).

Table. P2-2. H^+-jump experiments in mesophyll chloroplasts of *P. miliaceum*, a Na^+-type species

MCpt was suspended in a medium containing 5 mM Hepes-buffer (pH 8.2). Na^+- or H^+-jump were performed as described below.

The reaction time was 10 s by the single layer system.

		$[pyr]_{in}$ (mM)	ΔpH (In – Out)
Pyruvate uptake* (10 s)			
Dark	Control**	0.08±0	–1.2
	Na^+-jump (10 mM)	0.52±0.01	–1.2
	H^+-jump A (pH 8.2 to 7.4)	0.07±0.02	–0.3
	H^+-jump B (pH 8.2 to 7.0)	0.26±0.02	+0.1
	H^+-jump C (pH 8.2 to 6.7)	0.21±0.03	+0.4
Light	Control	0.67±0.03	–0.2
	Na^+-jump (10 mM)	1.39±0.03	–0.2
	H^+-jump A (pH 8.2 to 7.4)	0.58±0.04	+0.6
	H^+-jump B (pH 8.2 to 7.0)	0.48±0.04	+1.0
	H^+-jump C (pH 8.2 to 6.7)	0.41±0.06	+1.3
Stromal pH			
Dark	Control	7.10±0.05	
Light	Control	8.03±0.03	

* ; $[pyr]_{ex}$ = 1.0 mM

** ; Control; + 3.0 μl of DW
Na^+-jump; + 2.0 μl of 1.0 M NaCl
H^+-jump A; + 2.0 μl of 0.2 N HCl
H^+-jump B; + 3.0 μl of 0.2 N HCl
H^+-jump C; + 3.5 μl of 0.2 N HCl

Effects of illumination and Na^+-jump on ATP content in mesophyll chloroplasts from *P. miliaceum*

If Na^+ release from MCpt is driven by ATP formed in the stroma by illumination, Na^+ addition to the medium in the dark (Na^+-jump) might induce ATP synthesis by a mechanism of Na^+ pump found in some bacteria (Heefner and Harold 1980a, b). Actually, ATP content in MCpt of *P. miliaceum* was increased from about 5 to about 20 nmol / mg Chl by illumination (Table P2-3), which is comparable to spinach chloroplasts (from about 15 to 40 nmol / mg Chl, Kobayashi et al. 1979). However, Na^+-jump in the dark did not alter the ATP level in MCpt of *P. miliaceum* at the external pH of 7.0, 7.8 and 8.6 (Table P2-3). Similar results were obtained in the light or in the presence of $MgCl_2$ which was generally required for the reaction of ATP synthesis (Table P2-3).

Effect of exogenous Na^+ on the rate of pyruvate uptake into mesophyll chloroplasts from *P. miliaceum*

Ohnishi et al. (1990) reported that the chloroplast suspension from *P. miliaceum* usually contained 100 to 200 μM Na^+ due to a carry over by the medium. Fig. P2-5 shows the dependency on exogenously added Na^+ concentration of the rate of pyruvate uptake at external pH of 7.0 (a) or 7.8 (b), measured by the double layer system. In the experiments of Na^+-jump, NaCl was added only in the layer containing radioisotopes (the uptake layer) while NaCl was added both in the uptake layer and the chloroplast layer in the experiments in the presence of Na^+. Pyruvate uptake in the dark was not observed even in the presence of 10 mM NaCl either at pH 7.0 or at pH 7.8, while the activity was induced by Na^+-jump. At pH 7.8, pyruvate uptake in the dark was also induced by illumination either in Na^+-jump or in the presence of Na^+ (Fig. P2-5b), although a very little effect of light was observed at pH 7.0 (Fig.

Table P2-3. Effect of Na^+-jump on the ATP level in mesophyll chloroplasts of *P. miliaceum*

The MCpt precipitate after silicone oil centrifugation was used to measure the ATP content.

Conditions		ATP content (nmol / mg Chl)			
		Dark		Light	
		Control	Na^+-jump*	Control	Na^+-jump*
Exp. 1	pH 7.0	4.8	4.8	28.6	n.d.
	pH 7.8	4.5	4.6	25.0	n.d.
	pH 8.6	4.5	4.5	24.7	n.d.
Exp. 2	pH 7.8	5.2	5.2	20.9	n.d.
	pH 7.8, + 5 mM $MgCl_2$	4.8	4.7	20.2	n.d.
Exp. 3	pH 7.8	3.9	3.9	21.2	19.8

* ; 10 mM NaCl at final concentration. n.d. ; not determined.

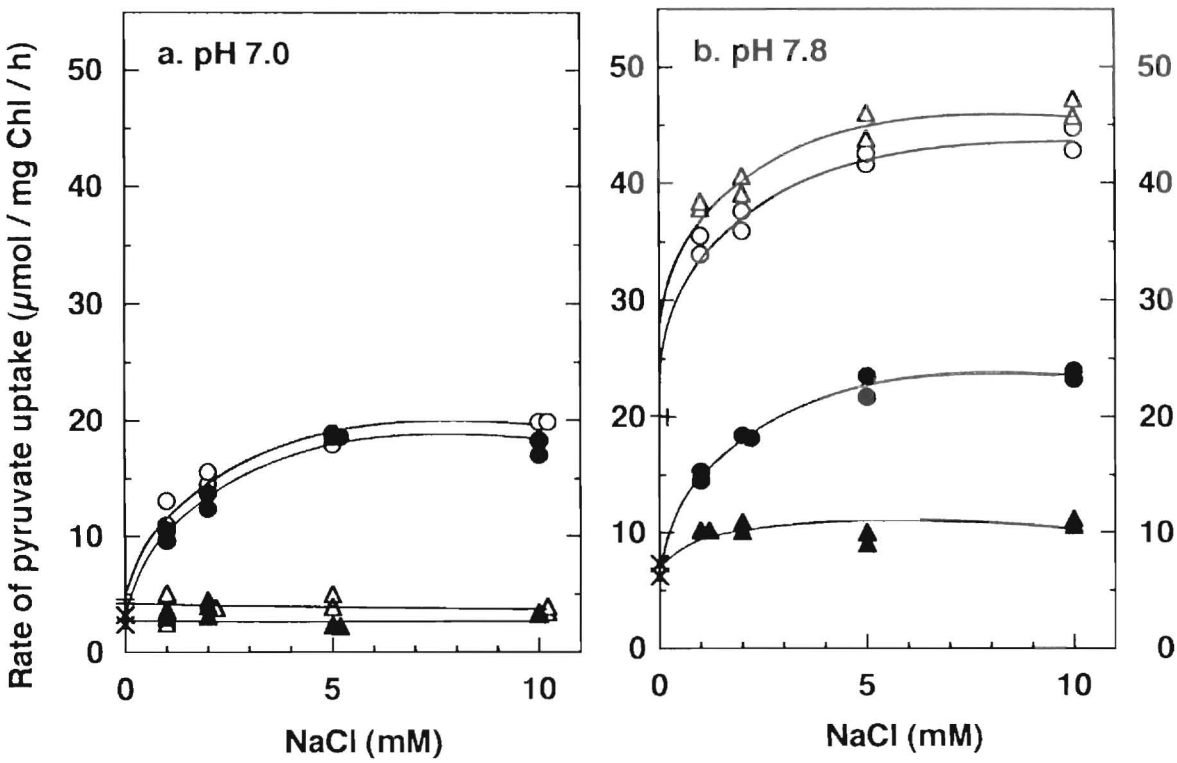


Fig. P2-5. The dependency on Na^+ concentration of the rate of pyruvate uptake into mesophyll chloroplasts of *P. miliaceum*

The rates of pyruvate uptake were measured both in the dark (x, ● and ▲) and in the light (x, o and Δ) at external pH of 7.0 (a) or 7.8 (b). In Na^+-jump experiments (● and o), Na^+ was added to the uptake layer of the double layer system. Experiments in the presence of Na^+ (▲ and Δ), Na^+ was added to both the uptake layer and the chloroplast layer. The reaction time was 2 s.

P2-5a). These activities which were induced by Na^+-jump and illumination showed similar pattern against the NaCl concentration, maximum rate at about 5 mM. As pyruvate uptake activity in the dark was enhanced by illumination without exogenous addition of Na^+ at pH 7.8 (Fig. P2-5), endogenous Na^+ in the medium may contribute considerably to form Na^+ gradient across the envelope to drive light-dependent pyruvate uptake into MCpt of *P. miliaceum*.

Discussion

Light-dependent pyruvate uptake into mesophyll chloroplasts of *P. miliaceum* is correlated with stromal alkalization by illumination

By the comparison of pyruvate uptake capacity formed by preillumination and light-dependent changes of stromal pH and ATP content in MCpt from *P. miliaceum*, Ohnishi and Kanai (1987b) concluded that the active pyruvate uptake in the light is primarily driven by the H^+ gradient across the envelope, but not by ATP in the stroma. Changes in the activity of light-dependent pyruvate uptake at external alkaline pH were related with that stromal pH difference between the light and the dark in MCpt of *P. miliaceum*, but not with ΔpH formed across the envelope in the light (Fig. P2-2). Furthermore, high activity of pyruvate uptake was achieved only when stromal pH was alkalized to about 7.8 or more in the light (Fig. P2-3). These results suggest that light-dependent pyruvate uptake into MCpt of Na^+-type C_4 species is induced mainly by stromal alkalization due to the activity of photosynthetic electron transport system in the thylakoid, but less by H^+ gradient formed across the envelope in the light.

If Na^+ gradient across the envelope is formed by illumination to drive the active pyruvate uptake into mesophyll chloroplasts of Na^+-type C_4 species?

In Na^+-type C_4 species such as *P. miliaceum*, it has been thought that the driving force of active pyruvate uptake into MCpt in the light is Na^+ gradient across the envelope, because light-dependent active pyruvate uptake is mimicked by Na^+-jump in the dark and pyruvate is cotransported with Na^+ in the ratio of 1 (Ohnishi et al. 1990). In this report, they showed, however, there is no evidence for the formation of Na^+ gradient across the envelope in illuminated MCpt of Na^+-type C_4 species. They also showed that by use of $^{22}Na^+$, Na^+ level in the stroma of MCpt of *P. miliaceum* was declined by illumination in some extent, but the lowered level was still higher than that in external medium (*cf.* Figure 2 in Ohnishi et al. 1990). As shown in Fig. P2-5, activities of pyruvate uptake in the presence of low level of Na^+ (ca. 0.5 mM) were remarkably enhanced by illumination only at external pH of 7.8. This seems to support that Na^+ gradient, if any, formed across the envelope in the light at external alkaline pH, not at neutral pH, would be a possible candidate for driving force of pyruvate uptake.

Possible mechanisms of the formation of Na^+ gradient across the envelope of mesophyll chloroplasts in the light

To form Na^+ gradient across the envelope of MCpt, the stromal Na^+ would be released to the cytosol or taken up into the thylakoid lumen. However, the latter possibility cannot explain continuous maintenance of the Na^+ gradient across the envelope during photosynthesis *in vivo*. Furthermore, Hind et al. (1974) reported in isolated thylakoid from spinach, a C_3 plants, that the distribution of Na^+ is essentially unaffected by illumination, using a Na^+ electrode.

As shown in Figure P2-1b and Table P2-3, stromal pH and the amount of ATP in MCpt of *P. miliaceum* increased by illumination. Since stromal alkalization by illumination is correlated with light-dependent pyruvate uptake, ΔpH across the envelope and/or stromal ATP are candidate(s) of the energy source for the formation of Na^+ gradient to drive pyruvate uptake.

Firstly, if a Na^+/H^+ antiporter is present in the envelope, ΔpH between stroma and cytosol would become a driving energy for release of Na^+ in MCpt of Na^+-type C_4 species. Na^+/H^+ antiporters are well known in membranes of various organisms and electrochemical potential of H^+ ($\Delta\mu_{H^+}$) is generally used for the driving force in exchange of Na^+ with H^+ (Padan and Schuldiner 1994). However, the results on the external pH dependency of light-dependent pyruvate uptake were not correspondent with that of ΔpH formed across the envelope in the light (Fig. P2-2). Furthermore, in MCpt of *P. miliaceum*, ΔpH formed across the envelope in the light was only about 0.5 units at most in the range of external pH from 7.0 to 8.6 (Fig. P2-1c), while in maize, it was more than 1.0 unit at pH 7.0 (Fig. P1-7c). These results together with independency of Na^+-jump on external pH suggest that main contribution of Na^+/H^+ antiporter to form Na^+ gradient across the envelope is unlikely in light-dependent active pyruvate uptake into MCpt of *P. miliaceum*, a Na^+-type C_4 species. Nevertheless, some role of Na^+/H^+ antiporter is not negated, since H^+-jump could induce in some extent pyruvate uptake into MCpt of *P. miliaceum* in the light (Table P2-2). Furthermore, not only the pH difference across the envelope but also the membrane potential are the components of $\Delta\mu_{H^+}$. Actually, Ohnishi and Kanai (1987b) reported that pyruvate uptake into MCpt of *P. miliaceum* was partly inhibited by 5 mM tetraphenylphosphonium, which is thought to reduce the membrane potential. This may suggest some contribution of the membrane potential to the formation of Na^+ gradient across the envelope.

Secondly, as light-dependent pyruvate uptake was higher at alkaline pH even above 8.0 in MCpt of *P. miliaceum* (Fig. P2-1), it was expected that Na^+ might be released across the envelope by a Na^+-translocating adenosine-5'-triphosphatase (Na^+-ATPase), which was reported in the plasmamembrane of a enterobacterium *Enterococcus hirae* (*Streptococcus faecalis*, Heefner and Harold 1980a, b). This organism has a Na^+/H^+ antiporter as well as an Na^+-ATPase. Both systems have been implied for Na^+ excretion from the bacterium; the Na^+/H^+ antiporter from acidic to neutral pH where $\Delta\mu_{H^+}$ is sufficient, whereas the Na^+-ATPase at alkaline pH where $\Delta\mu_{H^+}$ is limiting. So far, the artificial Na^+ gradient across the envelope could not induce ATP synthesis in MCpt of *P. miliaceum* (Table P2-3). The result would negate a possible role of Na^+-ATPase in MCpt of Na^+-type C_4 species such as *P. miliaceum*. Still remaining to study is the contribution of other ATPases, for example, Na^+/K^+-ATPase which is well known in plasmamembrane of various animal cells, although K^+ did not induce pyruvate uptake into MCpt of *P. miliaceum* in the dark (Ohnishi and Kanai 1987c).

In addition to the possibilities mentioned above, Na^+ gradient formed by any mechanism would be maintained, if PEP export via the Pi translocator could be accompanied with Na^+ to release it from MCpt of Na^+-type C_4 species, although PEP is transported together with H^+ in *P. miliaceum* (Table P1-3).

Main problem remained to be studied to understand the light-dependent active pyruvate uptake *in vivo* in mesophyll chloroplasts of Na^+-type C_4 species

It is generally thought that cytosolic pH is nearly neutral. However, the activity of light-dependent pyruvate uptake in MCpt of *P. miliaceum*, a Na^+-type C_4 species, is rather low at external pH of 7.0, compared with high activity at

alkaline pH (Fig. P2-1a). This makes difficult to implicate the physiological significance of light-dependent active pyruvate uptake at alkaline pH by MCpt of Na^+-type C_4 species. Although pyruvate uptake was induced by Na^+-jump in the dark even at pH 7.0 (Fig. P2-1 and P2-5), Na^+ gradient across the envelope to drive Na^+/pyruvate cotransport could not be formed by illumination at neutral pH. On the other hand, it should be noted that pyruvate uptake activity caused by Na^+-jump in the dark is quite similar to that in the light; no synergistic effect of illumination and Na^+-jump on pyruvate uptake was observed at neutral pH. Therefore, a synergistic effect seen in alkaline medium might be physiologically insignificant. To solve this and other problems, further experiments are required in use of $^{22}Na^+$ to estimate the actual change of stromal Na^+ concentration by illumination.

References

Aoki, N., Ohnishi, J. Kanai, R. (1992) Two different mechanisms for transport of pyruvate into mesophyll chloroplasts of C_4 plants—a comparative study. Plant Cell Physiol. 33: 805-809.

Edwards, G.E. and Walker, D.A. (1983) C_3, C_4: Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis. pp. 326-367. Blackwell Scientific Publications, Oxford.

Fliege, R., Flügge, U.-I., Werdan, K. and Heldt, H.W. (1978) Specific transport of inorganic phosphate, 3-phosphoglycerate and triosephosphates across the inner membrane of the envelope in spinach chloroplasts. Biochim. Biophys. Acta 502: 232-247.

Flügge, U.I., Stitt, M. and Heldt, H.W. (1985) Light-driven uptake of pyruvate into mesophyll chloroplasts from maize. FEBS Lett. 183: 335-339.

Flügge, U.I. and Heldt, H.W. (1986) Chloroplast phosphate-triosephosphate-phosphoglycerate translocator: Its identification, isolation and reconstitution. Methods Enzymol. 125: 716-730.

Flügge, U.-I. and Heldt, H.W. (1991) Metabolite translocators of the chloroplast envelope. Annu. Rev. Plant Physiol. Plant Molec. Biol. 42: 129-144.

Gross, A., Brückner, G., Heldt, H.W. and Flügge, U.-I. (1990) Comparison of the kinetic properties, inhibition and labelling of the phosphate translocators from maize and spinach mesophyll chloroplasts. Planta 180: 262-271.

Halestrap, A.P. (1975) The mitochondrial pyruvate carrier. Kinetics and specificity for substrates and inhibitors. Biochem. J. 148: 85-96.

Heefner, D.L. and Harold, F.M. (1980a) ATP-linked sodium transport in *Streptococcus faecalis*. I. The sodium circulation. J. Biol. Chem. 255: 11396-11402.

Heefner, D.L. and Harold, F.M. (1980b) ATP-linked sodium transport in *Streptococcus faecalis*. II. Energy coupling in everted membrane vesicles. J. Biol. Chem. 255: 11403-11407.

Heldt, H.W. (1980) Measurement of metabolite movement across the envelope and of the pH in the stroma and thylakoid space in intact chloroplasts. Methods Enzymol. 69: 604-613.

Hind, G., Nakatani, H.Y. and Izawa, S. (1974) Light-dependent redistribution of ions in suspensions of chloroplast thylakoid membranes. Proc. Nat. Acad. Sci. USA 71: 1484-1488.

Howitz, K.T. and McCarty, R.E. (1985) Kinetic characteristics of the chloroplast envelope glycolate transporter. Biochemistry 24: 2645-2652.

Huber, S.C. and Edwards, G.E. (1977a) Transport in C_4 mesophyll chloroplasts. Characterization of the pyruvate carrier. Biochim. Biophys. Acta 462: 583-602.

Huber, S.C. and Edwards, G.E. (1977b) Transport in C_4 mesophyll chloroplasts. Evidence for an exchange of inorganic phosphate and phosphoenolpyruvate. Biochim. Biophys. Acta 462: 603-612.

Jencks, W.P. and Regenstein, J. (1968) Ionization constants of acids and bases. In Handbook of Biochemistry. Selected Data for Molecular Biology. Edited by Sober, H.A. pp. J-150-189. The Chemical Rubber Co., Cleveland.

Kanai, R. and Edwards, G.E. (1973) Separation of mesophyll protoplasts and bundle sheath cells from maize leaves for photosynthetic studies. Plant Physiol. 51: 1133-1137.

Kobayashi, Y., Inoue, Y., Shibata, K. and Heber, U. (1979) Control of electron flow in intact chloroplasts by the intrathylakoid pH, not by the phosphorylation potential. Planta 146: 481-486.

Ohnishi, J. and Kanai, R. (1983) Differentiation of photorespiratory activity between mesophyll and bundle sheath cells of C_4 plants. I. Glycine oxidation by mitochondria. Plant Cell Physiol. 24: 1411-1420.

Ohnishi, J. and Kanai, R. (1987a) Pyruvate uptake by mesophyll and bundle sheath chloroplasts of a C_4 plant, *Panicum miliaceum* L. Plant Cell Physiol. 28: 1-10.

Ohnishi, J. and Kanai, R. (1987b) Light-dependent uptake of pyruvate by mesophyll chloroplasts of a C_4 plant, *Panicum miliaceum* L. Plant Cell Physiol. 28: 243-251.

Ohnishi, J. and Kanai, R. (1987c) Na^+-induced uptake of pyruvate into mesophyll chloroplasts of a C_4 plant, *Panicum miliaceum*. FEBS Lett. 219: 347-350.

Ohnishi, J. and Kanai, R. (1990) Pyruvate uptake induced by a pH jump in mesophyll chloroplasts of maize and sorghum, NADP-malic enzyme type C_4 species. FEBS Lett. 269: 122-124.

Ohnishi, J., Flügge, U.-I., Heldt, H.W. and Kanai, R. (1990) Involvement of Na^+ in active uptake of pyruvate in mesophyll chloroplasts of some C_4 plants. Na^+/pyruvate cotransport. Plant Physiol. 94: 950-959.

Ohnishi, J., Aoki, N. and Kanai, R. (1992) Pyruvate transport into mesophyll chloroplasts of various C_4 plants. In Research in Photosynthesis, Vol 3. Edited by Murata, N. pp. 737-740. Kluwer Academic Publishers, Dordrecht.

Padan, E. and Schuldiner, S. (1994) Molecular physiology of Na^+/H^+ antiporters, key transporters in circulation of Na^+ and H^+ in cells. Biochim. Biophys. Acta 1185: 129-151.

Rumpho, M.E. and Edwards, G.E. (1985) Characterization of 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene inhibition of 3-phosphoglycerate-dependent O_2 evolution in isolated chloroplasts. Evidence for a common binding site on the C_4 phosphate translocator for 3-phosphoglycerate, phosphoenolpyruvate, and inorganic phosphate. Plant Physiol. 78: 537-544.

Strehler, B.L. (1974) Adenosine-5'-triphosphate and creatine phosphate. Determination with luciferase. In Methods of Enzymatic Analysis, Vol 4. Edited by Bergmeyer, H.U. pp. 2112-2126. Verlag Chemie, Weinheim.

Sttit, M. and Heldt, H.W. (1985) Generation and maintenance of concentration gradients between the mesophyll and bundle sheath in maize leaves. Biochim. Biophys. Acta 808: 400-414.

Wintermans, J.F.G.M. and De Mots, A. (1965) Spectrophotometric characteristics of chlorophylls and their pheophytins in ethanol. Biochim. Biophys. Acta 109: 448-453.

Acknowledgement

卒業研究、修士課程、そして博士課程の計6年間にわたって、未熟な私をご指導いただき、学問に対する真摯な姿勢、研究に取り組む際の心構え、成果をあげる楽しさなどを教えて下さいました金井龍二教授に心から感謝いたします。ありがとうございました。

細胞生化学研究室での日常生活をはじめとしてセミナー、実験、また、学位論文の作成などに様々なアドバイスをいただきました大西純一先生、是枝晋先生、ありがとうございました。

博士課程の副指導教官になっていただき、様々なご助言や、あたたかい励ましをいただきました橋本洋一教授、檜山哲夫教授、松島久教授に深く感謝いたします。

分子生物学科の諸先生方、また事務の方には、多方面にわたりたいへんお世話になりました。この場を借りて感謝いたします。

最後に、埼玉大学理学部生化学科に入学してからの９年間、苦楽を共にし、良き遊び相手であり、良きライバルであり、また良き相談相手でもあった、野中隆君と三沢宏之君に深く感謝いたします。

青木直大