Effect of sulphate on glutamate synthesis by intact spinach (*Spinacia oleracea*) chloroplasts

Renaud DUMAS, Jacques JOYARD and Roland DOUCE†

Laboratoire mixte CNRS/Rhône-Poulenc (Unité Associée au Centre National de la Recherche Scientifique n° UM 41), Rhône-Poulenc Agrochimie, 14–20 rue Pierre Baizet, 69009 Lyon, France

During the course of NH_4^+ (or NO_2^-)-plus- α -oxoglutarate-dependent O_2 evolution in spinach (*Spinacia oleracea*) chloroplasts, glutamate was continuously excreted out of the chloroplasts. Under these conditions, for each molecule of NO_2^- or NH_4^+ which disappeared, one molecule of glutamate accumulated in the medium and the concentration of glutamate in the stroma space was maintained constant. SO_4^{2-} (or SO_3^{2-}) behave as inhibitors of NH_4^+ incorporation into glutamate by intact chloroplasts. This considerable inhibition of glutamate synthesis by SO_4^{2-} was correlated with a rapid decline in the stromal P_i concentration. The reloading of stromal P_i with either external P_i or PP_i^{4-} relieved SO_4^{2-} -induced inhibition of glutamate synthesis by intact chloroplasts. It was concluded that SO_4^{2-} induced a rapid efflux of stromal P_i out of the chloroplast, leading to a limitation of ATP synthesis and therefore to an arrest of ATP-dependent glutamine synthetase functioning.

INTRODUCTION

Illuminated chloroplasts catalyse in an 8e^- reduction the formation of glutamate from α -oxoglutarate in the presence of exogenous NO_2^- (Beevers & Hageman, 1980; Miflin & Lea, 1980). This reaction has been shown to be directly linked to ATP-dependent glutamine synthetase, reduced ferredoxin-dependent glutamate synthase and nitrite reductase activities (Beevers & Hageman, 1980; Miflin & Lea, 1980). Several important studies have also indicated that illuminated intact chloroplasts effect the reduction of SO_4^{2-} to S^{2-} in an 8e^- reduction using reduced ferredoxin as electron donor (Anderson, 1980; Giovanelli *et al.*, 1980). Under these conditions, it is possible that SO_4^{2-} and NO_2^- in the stroma space compete for their respective reductions at the level of reduced ferredoxin. We therefore set out to examine in detail the effect of SO_4^{2-} on glutamate excretion during the course of NO_2^- or NH_4^+ assimilation by isolated spinach (*Spinacia oleracea*) chloroplasts and have found evidence that SO_4^{2-} does inhibit this excretion. However, the competition does not exist, and our results are best explained by the operation of a sulphate carrier in the chloroplast envelope (Mouriaux & Douce, 1979), leading to a massive P_i efflux from intact isolated chloroplasts and therefore to an arrest of ATP-dependent glutamine synthetase functioning. In addition our results shed new light on the uptake of SO_4^{2-} and PP_i^{4-} by isolated chloroplasts.

MATERIALS AND METHODS

Isolation of chloroplasts

Young spinach (*Spinacia oleracea* L.) leaves (3 months old) were cut into small pieces directly into ice-cold chloroplast extraction medium [330 mM-sorbitol/50 mM-Hepes/KOH (pH 7.8)/2 mM-EDTA/0.15% (w/v) bov-

ine serum albumin] with a tissue/medium volume ratio of 1:3. Leaves were homogenized three times for 2 s each with a Waring Blendor, and intact chloroplasts were prepared as quickly as possible by the method of Nakatani & Barber (1977). Chloroplasts thus obtained were purified by isopycnic centrifugation in a non-toxic silica sol (Percoll, Pharmacia) gradient, which maintained isosmotic conditions throughout the isolation procedure, which was that described by Mouriaux & Douce (1981). The entire isolation procedure could be accomplished in less than 30 min. At this point in the procedure, the thick chloroplast suspension was stored in an ice bath under dark conditions.

Photosynthesis assay

O_2 evolution was followed polarographically at 25 °C by using a Clark-type-oxygen-electrode system (Delieu & Walker, 1972) purchased from Hansatech (King's Lynn, Norfolk, U.K.). The basic reaction medium contained, in a total volume of 1 ml, 330 mM-sorbitol, 50 mM-Hepes/KOH buffer, pH 7.8, and 5 mM- MgCl_2 . The reaction medium was gassed with argon before the addition of chloroplasts equivalent to 100–200 μg of Chl (i.e. 2–4 mg of protein). Reaction mixtures for the determination of CO_2 -dependent O_2 evolution contained 25 μM - K_2HPO_4 and 5 mM- NaHCO_3 . Reaction mixtures for the determination of NO_2^- (or NH_4^+)-plus- α -oxoglutarate-dependent O_2 evolution contained 2.5 mM- α -oxoglutarate, 2 mM-malate and known amounts of NO_2^- or NH_4^+ . The reaction was triggered once CO_2 -dependent O_2 evolution had ceased. The light was provided by a 150 W xenon arc-lamp source (Oriol Corporation) giving an irradiance of 300 $\text{W}\cdot\text{m}^{-2}$ at the surface of the vessel.

Conditions for the determination of O_2 evolution by the reconstituted chloroplast system in the presence of NH_3 , glutamate and α -oxoglutarate were adapted from those described by Anderson & Walker (1983). All

Abbreviation used: Chl, chlorophyll.

† To whom correspondence and reprint requests should be sent.

reactions were allowed to proceed in the basic medium containing 2 mM-ATP, 4 mM- K_2HPO_4 , 4 mM-ascorbate, 1 mM-dithiothreitol, 0.1 mM-NADPH, ferredoxin ($50 \mu\text{g}\cdot\text{ml}^{-1}$), catalase ($50 \text{ units}\cdot\text{ml}^{-1}$) and osmotically shocked chloroplasts ($150 \mu\text{g}$ of $\text{Chl}\cdot\text{ml}^{-1}$).

Glutamate determination

Chloroplast suspension (1 ml, 100–200 μg of Chl) was centrifuged for 2 min at 1000 g (Eppendorf centrifuge 5415). The supernatant containing excreted glutamate (S_1) was removed by aspiration and the pellet was resuspended in 300 μl of water/trichloroacetic acid (100:1, v/v). After sonication, the suspension was centrifuged for 3 min at 16000 g (Eppendorf, centrifuge 5415). The supernatant thus obtained (S_2) contained glutamate molecules sequestered in the stroma space.

Derivatization of amino acids with phenyl isothiocyanate. Aliquots (20 μl) of S_1 and S_2 were evaporated under vacuum. Samples were neutralized after adding 20 μl of ethanol/water/triethylamine (1:2:2, by vol.) and again evaporated to dryness. The samples thus obtained were solubilized with 10 μl of water/ethanol (1:1, v/v) and ready for derivatization. The derivatization reagent was made fresh daily and consisted of ethanol/triethylamine/water/phenyl isothiocyanate (7:1:1:1, by vol.). Phenylthiocarbonyl derivatives of the amino acids were allowed to form for 20 min at room temperature after 50 μl of reagent had been added to the solubilized samples. The derivatization reagents were then removed under vacuum and the amino acid derivatives were dissolved in 1 ml of medium containing 4 mM- KH_2PO_4 (pH 7.5) and 6% (v/v) acetonitrile (Bidlingmeyer *et al.*, 1984).

Chromatography. The system was a Millipore liquid chromatograph which consisted of two Waters model-510 h.p.l.c. pumps and a M440 fixed-wavelength detector (254 nm). The temperature (39 $^{\circ}\text{C}$) was controlled within $\pm 1^{\circ}\text{C}$ with a column heater (Waters). The column was application-specified Pico-Tag column, packed in 15 cm $\times$ 3.9 mm hardware, and quality-controlled for rapid, high-efficiency, bonded-phase separation. The column, equipped with a Guard-PAK (Waters) pre-column insert, was previously equilibrated with eluent A [0.14 M-sodium acetate/4 mM-triethylamine (adjusted to pH 6.35 with acetic acid)]. Samples were injected in a volume of 50 μl (0.05–0.5 nmol of glutamate) using a M710B WISP auto injector (Waters). The gradient run for the separation consisted of 90% eluent A + 10% eluent B [60% (v/v) acetonitrile in water] traversing to 49% eluent A + 51% eluent B in 13 min (flow rate 1 $\text{ml}\cdot\text{min}^{-1}$) using a convex curve ($n^{\circ} 5$) programmed with a M680 automated gradient controller (Waters). Peak integration was carried out with a single-channel recorder/integrator (Waters 740 data module). To determine accurately the amount of glutamate in the samples, a calibration of the glutamate peak with known amounts of external derivatized glutamate was first performed.

P_i determination

P_i measurement was made with intact chloroplasts (100–150 μg of Chl/ml) illuminated in the basic reaction medium containing various anions (SO_4^{2-} , PP_i^{4-}).

Samples (1 ml) removed at 1 min intervals were immediately centrifuged at 3 $^{\circ}\text{C}$ for 1 min at 1500 g in 2 ml basic reaction medium containing 0.4 M-mannitol to separate the chloroplasts from the medium. The supernatant was discarded by aspiration and the pellet was resuspended in 5% trichloroacetic acid (1 ml). The suspension was centrifuged 10 min at 7000 g (Sorvall, SM 24 rotor) and the clear supernatant, devoid of mannitol, which interferes with phosphomolybdate-complex formation, was used for the colorimetric determination of P_i after butan-2-ol extraction by the method of Yanagita (1964).

RESULTS

Glutamate synthesis by intact spinach chloroplasts

Isolated intact chloroplasts illuminated in a medium with optimal concentrations of α -oxoglutarate (2.5 mM) and malate (2 mM) excreted glutamate in the presence of either NO_2^- or NH_4^+ (Fig. 1) at a maximal rate of 9 $\mu\text{mol}/\text{h}$ per mg of Chl , thus confirming the earlier reports of Anderson & Done (1977) and Woo & Osmond (1982). The absence of malate decreased this rate considerably (result not shown). After cessation of O_2 evolution there exists a linear relationship between the total amount of glutamate recovered in the external medium and the amount of NO_2^- or NH_4^+ added (from 50 to 300 μM) in the medium at the beginning of the experiment (Fig. 1); for each molecule of NO_2^- or NH_4^+ which disappeared, one molecule of glutamate accumulated in the bathing medium. During the course of NH_4^+

Fig. 1. Effect of concentration of NH_4^+ (A) and NO_2^- (B) on glutamate excretion by intact spinach chloroplasts

The reaction mixture was prepared as described in the Materials and methods section and contained chloroplasts equivalent to 200 μg of $\text{Chl}\cdot\text{ml}^{-1}$ and 2.5 mM- α -oxoglutarate. The reaction was started by the addition of 2 mM-malate (final vol. 1 ml) and NH_4^+ or NO_2^- as indicated. Incubations were conducted in the light. When O_2 evolution had ceased (after 5 min) the suspension was centrifuged (Eppendorf centrifuge 5415; 2 min; 1000 g) and glutamate was measured (see the Materials and methods section) in the pellet (endogenous glutamate) and the supernatant (excreted glutamate). α -Oxoglutarate triggered the formation of a small amount of glutamate (approx. 50 nmol) at the expense of an endogenous pool of aspartate and glutamine at the beginning of the experiment. We did not take glutamate formed by transamination into account in this experiment.

(or NO_2^-)-plus- α -oxoglutarate-dependent O_2 evolution in intact chloroplasts the concentration of glutamate in the stroma space ('Pellet', see Fig. 1) was maintained constant, suggesting, in agreement with Woo *et al.* (1987b), the existence of a non-exchangeable endogenous pool of glutamate (approx. 10 mM). Interestingly, during the course of NO_2^- -dependent O_2 evolution in the absence of α -oxoglutarate, we have observed that NH_3 diffused readily towards the bathing medium and did not accumulate inside the thylakoids (results not shown).

Effect of SO_4^{2-} on glutamate synthesis by intact spinach chloroplasts

Fig. 2 shows the effect of SO_4^{2-} on NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution in intact chloroplasts in the presence of optimal concentrations of malate. When SO_4^{2-} was supplied at 3.5 and 8 mM, the rate of NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution was inhibited by 50 and 90% respectively. Fig. 3 indicates that either SO_4^{2-} or SO_3^{2-} also strongly inhibited glutamate excretion by illuminated chloroplasts maintained in a medium containing α -oxoglutarate and NO_2^- . Similar results were obtained with NH_4^+ instead of NO_2^- (results not shown). These results together strongly suggest, therefore, that SO_4^{2-} and SO_3^{2-} behave as inhibitors of NH_4^+ incorporation into glutamate by intact chloroplasts. This inhibition could be attributable to a direct effect of SO_4^{2-} or SO_3^{2-} on glutamine synthetase or glutamate synthase. This hypothesis, however, is not tenable, because neither SO_4^{2-} nor SO_3^{2-} added at 10 mM inhibited the rate of NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution catalysed by a reconstituted system prepared as described by Anderson & Walker (1983) (results not shown).

Mourioux & Douce (1979) have demonstrated that the transport of SO_4^{2-} and SO_3^{2-} into spinach chloroplasts

Fig. 2. Effect of SO_4^{2-} concentrations on NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution in intact spinach chloroplasts

The reaction mixture contained chloroplasts equivalent to 150 μg of $\text{Chl} \cdot \text{ml}^{-1}$, 2.5 mM- α -oxoglutarate, 2 mM-malate and 100 μM - NH_4Cl . All other conditions were as described in the Materials and methods section.

Fig. 3. Effect of concentration of SO_4^{2-} (A) and SO_3^{2-} (B) on glutamate excretion by intact spinach chloroplasts

The reaction mixture contained chloroplasts equivalent to 200 μg of $\text{Chl} \cdot \text{ml}^{-1}$ and 2.5 mM- α -oxoglutarate. The reaction was started by addition of 2 mM-malate and 100 μM - NO_2^- and SO_4^{2-} or SO_3^{2-} as indicated (final volume 1 ml). Incubations were conducted in the light. When O_2 evolution had ceased (after 5 min) the suspension was centrifuged (Eppendorf centrifuge 5415; 2 min, 1000 g) and glutamate was measured (see the Materials and methods section) in the pellet (endogenous glutamate) and the supernatant (excreted glutamate). α -Oxoglutarate triggered the formation of a small amount of glutamate (50 nmol) at the expense of an endogenous pool of aspartate and glutamine at the beginning of the experiment. We did not take glutamate formed by transamination into account in this experiment.

occurred by a strict counter-exchange; for each molecule of SO_4^{2-} entering the chloroplast, one molecule of HPO_4^{2-} leaves the stroma and vice versa. Under these conditions, such a transport would decrease stromal P_i to the point where it limits ATP synthesis. This in turn would rapidly inhibit ATP-dependent glutamine synthetase activity. To verify this hypothesis we have studied the effects of SO_4^{2-} -dependent depletion of stromal P_i on NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution and glutamate excretion by intact chloroplasts. Assuming a chloroplast volume of 25 $\mu\text{l}/\text{mg}$ of Chl (Heldt, 1980) the concentration of P_i found in the stroma of freshly prepared chloroplasts was 12 ± 3 mM (Table 1). Values between 4 and 20 mM have been reported (Kaiser & Urbach, 1977; Lilley *et al.*, 1977; Mourioux & Douce, 1981; Furbank *et al.*, 1987). Table 1 shows that the stromal P_i concentration rapidly decreased from approx. 12 mM in the absence of SO_4^{2-} to a constant steady-state concentration of between 1 and 1.2 mM in the presence of 4 mM- SO_4^{2-} . These results together demonstrate that P_i effluxed rapidly from intact isolated chloroplasts maintained in a medium containing 4 mM- SO_4^{2-} .

The stromal P_i concentration attained at the equi-

Table 1. Time course of changes in stromal P_i content in intact chloroplasts incubated with 4 mM- Na_2SO_4

The reaction mixture and P_i determination were as described in the Materials and methods section. Assuming a chloroplast volume of 25 $\mu\text{l}/\text{mg}$ of Chl (Heldt, 1980), the concentration of P_i found in the stroma of freshly prepared chloroplasts was approx. 12 mM.

Time (min)	Stromal [P_i] (nmol $\cdot$ mg of Chl $^{-1}$)
0	300
0.5	140
1	80
2	50
4	30
8	35

Table 2. Steady-state stromal P_i concentration in chloroplasts maintained for 5 min in a medium containing various amounts of SO_4^{2-}

The reaction mixture and P_i determination were as described in the Materials and methods section.

[SO_4^{2-}] (mM)	Stromal [P_i] (mM)
0	12
1	9
2	5
4	1.2
8	0.8

librium decreased as SO_4^{2-} concentration increased in the suspension medium up to 6 mM (Table 2). The stromal P_i concentration thus obtained (approx. 0.8–1 mM) was not further diminished at higher SO_4^{2-} concentrations (Table 2). It appears that much of this pool was not readily available to the sulphate carrier located on the chloroplast inner membrane. In support of this suggestion, fairly recent evidence suggested that a small part of stromal P_i may be bound to ribulose 1,5-bisphosphate carboxylase (Furbank *et al.*, 1987) or associated with thylakoid membranes as a non-metabolic pool (Giersch & Robinson, 1987). Comparison between Table 2 and Fig. 2 indicates that there was an excellent correlation between the rate of NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution and the concentration of P_i in the stroma space obtained at various external SO_4^{2-} concentrations. Addition of P_i (300 μM) to the suspension medium restored the initial rate of NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution and glutamate excretion by intact chloroplasts (Fig. 4). Such a result indicates that P_i enters the chloroplast in exchange of SO_4^{2-} (Mouriaux & Douce, 1979). The resulting increase in stromal P_i restarts ATP synthesis and leads to a marked stimulation of ATP-dependent glutamine synthetase activity.

Interestingly, sulphate also strongly inhibited CO_2 -dependent O_2 evolution by intact chloroplasts, as shown previously by Baldry *et al.* (1968) (Fig. 5). However, in

Fig. 4. Inhibition by SO_4^{2-} of NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution and glutamate excretion in intact spinach chloroplasts and relief of these inhibitions by P_i

The reaction mixture was as described in the Materials and methods section and contained chloroplasts equivalent to 120 μg of Chl $\cdot$ ml $^{-1}$ and 2.5 mM- α -oxoglutarate (αKgl). The reactions were started by the addition of 2 mM-malate and 300 μM - NH_4Cl (final volume 1 ml). Incubations were conducted in the light. O_2 evolution was monitored polarographically at 25 $^\circ\text{C}$ by using a Clark-type oxygen-electrode system. At times indicated the suspension was centrifuged (Eppendorf centrifuge 5415; 2 min; 1000 g) and the glutamate excreted was measured in the supernatant (see the Materials and methods section). α -Oxoglutarate triggered the formation of a small amount of glutamate (30 nmol) at the expense of an endogenous pool of aspartate and glutamine at the beginning of the experiment. We did not take glutamate formed by transamination into account in this experiment. The broken lines indicate control experiments without SO_4^{2-} .

this case P_i alone did not restore the initial rate of CO_2 -dependent O_2 evolution. Moreover higher concentrations of P_i were found to be inhibitory because, as demonstrated by Walker (1967), an excess of P_i in the bathing medium led to a massive efflux of triose phosphate and, therefore, to an arrest of the Benson-Calvin cycle.

The fact that PP_i^{4-} also relieved the SO_4^{2-} inhibition of glutamate excretion by intact chloroplasts after a lag phase (results not shown) strongly suggests that PP_i^{4-} enters the chloroplast. Under these conditions the presence of a powerful alkaline pyrophosphatase activity in the stroma space (Gross & ap Rees, 1986) would increase stromal P_i concentration. Table 3 actually demonstrates that addition of 2 mM- PP_i^{4-} to P_i -depleted chloroplasts led to a marked increase in stromal P_i concentration. For example, the stromal P_i concentration increased from approx. 0.8 mM (P_i -deficient chloroplasts) to a concentration of approx. 6 mM after 4 min in the presence of 2 mM- PP_i^{4-} .

Fig. 5. Inhibition by SO_4^{2-} of CO_2 -dependent O_2 evolution in intact spinach chloroplasts and partial relief of this inhibition by PP_i^{4-} (a) or P_i (b) or $\text{P}_i + \text{PP}_i^{4-}$ (c)

The reaction mixture was as described in the Materials and methods section and contained chloroplasts equivalent to $150 \mu\text{g}$ of $\text{Chl} \cdot \text{ml}^{-1}$, $25 \mu\text{M}$ -phosphate and 5 mM - NaHCO_3 (final vol. 1 ml). Incubations were conducted in the light. O_2 evolution was monitored polarographically at 25°C by using a Clark-type oxygen-electrode system. L, light. Numbers on traces refer to μmol of O_2 evolved/h per mg of chl.

Table 3. Time course of changes in stromal P_i content in P_i -depleted intact chloroplasts incubated with 2 mM - PP_i^{4-}

The reaction mixture and P_i determination were as described in the Materials and methods section. P_i -depleted intact chloroplasts were obtained at room temperature as described by Mourioux & Douce (1981).

Time (min)	Stromal $[\text{P}_i]$ (nmol $\cdot \text{mg}$ of Chl^{-1})
0	50
1	100
2	170
4	240
8	290

DISCUSSION

The data reported here show that, during the course of NH_4^+ (or NO_2^-)-plus- α -oxoglutarate-dependent O_2 evolution in intact chloroplasts, glutamate was continuously excreted out of the chloroplasts. For each molecule of NO_2^- or NH_4^+ which disappeared, one molecule of glutamate accumulated in the bathing medium. These results are in good agreement with the original observations of Anderson & Done (1977); glutamate synthase and glutamine synthetase established a self-sustaining system for ammonia incorporation where glutamine

synthetase initiates the net synthesis of glutamate at the expense of α -oxoglutarate. The strong effect of malate on the rate of glutamate excretion we have observed is also in good agreement with the original observation of Woo & Osmond (1982), leading to the demonstration that α -oxoglutarate is transported inward on a α -oxoglutarate translocator and glutamate outward on a dicarboxylate translocator with malate as the counterion on both translocators in a cascade-like manner (Dry & Wiskich, 1983; Woo *et al.*, 1987a). Interestingly, in the absence of α -oxoglutarate we have shown that NH_4^+ deriving from NO_2^- reduction under light conditions did not accumulate inside the thylakoids, although a considerable ΔpH gradient is settled across the thylakoid membrane favouring the accumulation of NH_4^+ . Such a result strongly suggests that nitrite reductase operates near the periphery of the stroma space or in close contact with the inner envelope membrane, perhaps near the nitrite-permeation channel or transport protein (Brunswick & Cresswell, 1988), favouring the free diffusion of NH_3 out of the chloroplasts. Furthermore, under these conditions, NH_4^+ produced near the periphery may not be easily transported towards thylakoids because of stroma viscosity (0.4 g of protein per ml) (Douce & Joyard, 1979).

In the light, the considerable inhibition of glutamate synthesis by SO_4^{2-} in intact chloroplasts is correlated with a rapid decline in the stromal P_i concentration. The principal arguments in favour of this conclusion are numerous: (a) SO_4^{2-} did not affect the rate of NH_4^+ -plus- α -oxoglutarate-dependent O_2 evolution catalysed by a reconstituted system; (b) SO_4^{2-} induced a rapid efflux of stromal P_i out of the chloroplast via a specific sulphate carrier catalysing a strict counter-exchange: for each molecule of SO_4^{2-} entering the chloroplast one molecule of P_i leaves the stroma, and vice versa; (c) the reloading of stromal P_i with either external P_i or PP_i^{4-} relieved SO_4^{2-} -induced inhibition of glutamate synthesis of intact chloroplasts. It is clear, therefore, that a decrease in stromal P_i concentration would limit ATP synthesis (the chloroplast ATP synthase exhibits a rather low affinity for P_i ; Selman & Selman-Reimer, 1981), which would decrease the stromal ATP concentration and inhibit glutamine synthetase because the K_m values for ATP of angiosperm glutamine synthetases are rather low (0.8 – 1.3 mM) (Stewart *et al.*, 1980). Such a cascade of reactions would stop glutamate synthesis by intact chloroplasts.

Finally, the results presented here strongly suggest that PP_i^{4-} is transported through the chloroplast envelope. In support of this suggestion we have observed that PP_i^{4-} induced a marked increase in the stromal P_i concentration in P_i -depleted chloroplasts. It is possible that the driving force for the penetration of PP_i^{4-} through the chloroplast envelope is attributable to the presence of a powerful alkaline pyrophosphatase in the stromal space (Gross & ap Rees, 1986) which is involved in the rapid hydrolysis of PP_i^{4-} . This anion can enter the chloroplast either via a nucleotide carrier (Robinson & Wiskich, 1977) or via the P_i translocator (Fliege *et al.*, 1978). The first hypothesis is most unlikely, because PP_i^{4-} would decrease the nucleotide concentration in the stromal space, which would inhibit glutamine synthetase; obviously this was not the case. The second hypothesis does not explain the net accumulation of P_i in the stroma of intact chloroplasts maintained in a medium containing PP_i^{4-} . Furthermore, the results obtained by Fliege *et al.* (1978) were carried out using suspensions of unpurified

chloroplasts containing free pyrophosphatase deriving from broken chloroplasts. Under these conditions, exogenous P_i deriving from exogenous PP_i^{4-} hydrolysis would exchange with internal P_i . We are forced to imagine, therefore, that the chloroplast envelope possesses a PP_i^{4-} carrier involved in the net import of PP_i^{4-} . Obviously more work is required to characterize with absolute certainty this carrier, because it could be involved in the net accumulation of P_i in the stroma space and could play an important role in the coarse control of photosynthesis (Furbank *et al.*, 1987). Finally, the evidence that PP_i^{4-} can cross the chloroplast envelope very slowly is of particular importance in view of the recent demonstration that spinach leaves contain appreciable levels of PP_i^{4-} , which is confined to the cytosol (Weiner *et al.*, 1987).

We wish to thank Dr. Pierre Genix and Gilles Mourioux for their helpful suggestions.

REFERENCES

- Anderson, J. W. (1980) in the *Biochemistry of Plants*, vol. 5: Amino Acids and Derivatives (Mifflin, B. J., ed.), pp. 203–223, Academic Press, New York
- Anderson, J. W. & Done, J. (1977) *Plant Physiol.* **60**, 354–359
- Anderson, J. W. & Walker, D. A. (1983) *Planta* **159**, 77–83
- Baldry, C. W., Cockburn, W. & Walker, D. A. (1968) *Biochim. Biophys. Acta* **153**, 476–483
- Beevers, L. & Hageman, R. H. (1980) in *The Biochemistry of Plants*, vol. 5: Amino Acids and Derivatives (Mifflin, B. J., ed.), pp. 115–168, Academic Press, New York
- Bidlingmeyer, B. A., Cohen, S. A. & Tarvin, T. L. (1984) *J. Chromatogr.* **336**, 93–104
- Brunswick, P. & Cresswell, C. F. (1988) *Plant Physiol.* **86**, 378–383
- Delieu, T. & Walker, D. A. (1972) *New Phytol.* **71**, 201–225
- Douce, R. & Joyard, J. (1979) *Adv. Bot. Res.* **7**, 1–116
- Dry, I. B. & Wiskich, J. T. (1983) *Plant Physiol.* **72**, 291–296
- Fliege, R., Flügge, U. I., Werdam, K. & Heldt, H. W. (1978) *Biochim. Biophys. Acta* **502**, 232–247
- Furbank, R. T., Foyer, C. H. & Walker, D. A. (1987) *Biochim. Biophys. Acta* **894**, 522–561
- Giersch, C. & Robinson, S. P. (1987) *Photosynth. Res.* **14**, 211–227
- Giovanelli, J., Mudd, S. H. & Datko, A. H. (1980) in *The Biochemistry of Plants*, vol. 5: Amino Acids and Derivatives (Mifflin, B. J., ed.), pp. 453–505, Academic Press, New York
- Gross, P. & ap Rees, T. (1986) *Planta* **167**, 140–145
- Heldt, H. W. (1980) *Methods Enzymol.* **69**, 604–613
- Kaiser, W. & Urbach, W. (1977) *Biochim. Biophys. Acta* **459**, 337–346
- Lilley, R. Mc C., Chon, C. J., Mosbach, A. M. & Heldt, H. W. (1977) *Biochim. Biophys. Acta* **460**, 259–272
- Mifflin, B. J. & Lea, P. J. (1980) in *The Biochemistry of Plants*, vol. 5, Amino Acids and Derivatives (Mifflin, B. J., ed.), pp. 169–202, Academic Press, New York
- Mourioux, G. & Douce, R. (1979) *Biochimie* **61**, 1283–1292
- Mourioux, G. & Douce, R. (1981) *Plant Physiol.* **67**, 470–473
- Nakatani, H. Y. & Barber, J. (1977) *Biochim. Biophys. Acta* **461**, 510–512
- Robinson, S. P. & Wiskich, J. T. (1977) *Plant Physiol.* **59**, 422–427
- Selman, B. R. & Selman-Reimer, S. (1981) *J. Biol. Chem.* **256**, 1722–1726
- Stewart, G. R., Mann, A. F. & Fentem, P. A. (1980) in *The Biochemistry of Plants*, vol. 5: Amino Acids and Derivatives (Mifflin, B. J., ed.), pp. 271–327, Academic Press, New York
- Walker, D. A. (1967) in *The Biochemistry of the Chloroplasts*, vol. 2: Photosynthetic Activity of Isolated Pea Chloroplasts (Goodwin, T. W., ed.), pp. 53–69, Academic Press, New York
- Weiner, H., Stitt, M. & Heldt, H. W. (1987) *Biochim. Biophys. Acta* **893**, 13–21
- Woo, K. C. & Osmond, C. B. (1982) *Plant Physiol.* **69**, 591–596
- Woo, K. C., Flügge, U. I. & Heldt, H. W. (1987a) *Plant Physiol.* **84**, 624–632
- Woo, K. C., Boyle, F. A., Flügge, U. I. & Heldt, H. W. (1987b) *Plant Physiol.* **85**, 621–625
- Yanagita, T. (1964) *J. Biochem. (Tokyo)* **55**, 260–268

Received 5 August 1988/1 November 1988; accepted 14 November 1988