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TRANSPORT IN C_4 MESOPHYLL CHLOROPLASTS

EVIDENCE FOR AN EXCHANGE OF INORGANIC PHOSPHATE AND PHOSPHOENOLPYRUVATE

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SUMMARY

1. Mesophyll chloroplasts of the C_4 plant *Digitaria sanguinalis* contain endogenous phosphoenolpyruvate which appears to distribute across the envelope according to the existing pH gradient. The phosphoenolpyruvate remaining in the stroma can be rapidly released by external inorganic phosphate or 3-phosphoglycerate while external pyruvate did not affect the distribution.

2. Phosphoenolpyruvate (PEP) was a competitive inhibitor (K_i(PEP) = 450 μM) of $^{32}P_i$ uptake ($K_m(P_i)$ = 200 μM) by chloroplasts in the dark and also reduced the steady-state internal concentration of $^{32}P_i$, which is consistent with phosphate and phosphoenolpyruvate sharing a common carrier.

3. Phosphoenolpyruvate formation by chloroplasts in the light in the presence of pyruvate but in the absence of inorganic phosphate was slow and the concentration ratio of phosphoenolpyruvate (internal/external) was high. Addition of 0.1 mM phosphate induced a high rate of phosphoenolpyruvate formation and the concentration ratio (internal/external) decreased 15-fold. It is proposed that external phosphate is required both for phosphoenolpyruvate formation and efflux from the chloroplast.

INTRODUCTION

Localization studies of photosynthetic enzymes in C_4 plants indicate that enzymes for CO_2 fixation through the C_4 pathway are in the mesophyll cell while the enzymes for CO_2 fixation through the Calvin cycle are in the bundle sheath cells [1]. On the basis of such studies as well as studies with intact chloroplasts in vitro, a common function of the mesophyll chloroplasts of C_4 plants has been suggested to be the conversion of pyruvate to phosphoenolpyruvate within the stroma [2–4]. Schemes

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Abbreviations: C_3 plant, plant having only the Calvin-Benson pathway of photosynthesis; C_4 plant, plant having the Calvin-Benson pathway and the C_4-dicarboxylic acid pathway of photosynthesis; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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for the flow of carbon in the C_4 mesophyll cell propose that pyruvate is transported into the chloroplast where it is converted to phosphoenolpyruvate by pyruvate, orthophosphate dikinase. The phosphoenolpyruvate would then be transported out of the chloroplast to the cytosol for carboxylation by phosphoenolpyruvate carboxylase to form oxalacetate, the first product of the C_4 pathway. The inorganic phosphate released during the carboxylation of phosphoenolpyruvate would re-enter the chloroplasts. These schemes require the transport of pyruvate, P_i and phosphoenolpyruvate across the envelope of the C_4 mesophyll chloroplast. Direct evidence for carrier-mediated transport of pyruvate across the envelope of the C_4 plant *Digitaria sanguinalis* has been recently obtained [5]. To date, however, there has been no evidence to support the proposed transport of P_i and phosphoenolpyruvate in C_4 mesophyll chloroplasts. Furthermore, while the phosphate translocator of spinach (C_3) chloroplasts will readily transport P_i, phosphoenolpyruvate is a rather poor transport metabolite [6].

This communication reports experiments which suggest that a carrier exists in the envelope of the C_4 mesophyll chloroplast which facilitates an exchange-diffusion of P_i and phosphoenolpyruvate. The relation of this transport to that of pyruvate is discussed.

MATERIALS AND METHODS

Chemicals. Except as indicated, all reagents were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Silicone oil type F-50 was kindly supplied by General Electric, Silicon Products Division. $^{32}P_i$ (carrier free) was obtained from New England Nuclear.

Chloroplasts. Mesophyll chloroplasts (90–98 % intact on the basis of enzyme retention and ferricyanide exclusion) were obtained from leaves of *D. sanguinalis* as previously described [4]. Chloroplasts were generally resuspended in a medium containing 0.3 M sorbitol and 25 mM Tricine/KOH (pH 7.8).

Silicone oil centrigual filtration. Chloroplasts were rapidly separated from the incubation medium by centrifugation (10 000 × *g*, 15 s) through a 50 μl layer of silicone oil into a bottom layer of 20 μl of 7 % $HClO_4$, as previously described for spinach chloroplasts [7]. The 70 μl incubation medium typically contained 0.3 M sorbitol, 50 mM Tricine/Tris, 10 mM K_2SO_4, 10 μM $K_2H^{32}PO_4$ (1000 μCi/mol and chloroplasts (5–15 μg chlorophyll). Reactions were initiated by the addition of chloroplasts and terminated by centrifugation. After completion, a 20 μl aliquot of the top layer was counted in scintillation fluid to determine total dpm in the incubation mixture and the entire bottom layer was excised and counted in scintillation fluid to determine dpm in the chloroplast pellet. Quench correction was by external standard. The amount of label in the chloroplast pellet was corrected for non-osmotic uptake and absolute chloroplast volumes were determined by the uptake of ^3H_2O and [^{14}C] sucrose [7].

For analysis of phosphoenolpyruvate distribution between the stroma and medium (experiments of Figs. 1 and 5), chloroplasts were separated from the incubation mixture by centrifugation through silicone oil as described above. All experiments which involved analysis of phosphoenolpyruvate were performed with CO_2-free media to avoid any carboxylation of phosphoenolpyruvate by residual phosphoenolpyruvate carboxylase found as a contaminant of the chloroplast preparations (< 2 % of the total activity in the mesophyll cells). Sample preparations were as follows. Immediately after silicone oil centrifugation, the entire supernatant (top phase) was acidified with 10 μl 7 % $HClO_4$ followed by neutralization with 5 μl of a mixture containing 2.5 M KOH, 0.4 M HEPES, and 50 mM K_2HPO_4. The bottom layer (20 μl 7 % $HClO_4$) was also neutralized with the above mixture. After neutralization, the samples were clarified by centrifugation (10 000 × *g*, 1 min).

Analysis of phosphoenolpyruvate. Aliquots of the supernatant and chloroplast pellet samples were mixed with 50 μl of 10 mM KCl, 50 mM HEPES (pH 7.4), 8 mM $MgCl_2$, 3 μM ADP and two units of pyruvate kinase. After incubation for 20 min at 30 °C, the conversion enzyme was denatured by acidification followed by neutralization. The ATP in the samples (equivalent to the amount of phosphoenolpyruvate originally present) was then assayed by the luciferin-luciferase assay as previously described [8].

Phosphoenolpyruvate formation by intact chloroplasts. Chloroplasts were washed twice and resuspended in CO_2-free media containing 0.3 M sorbitol, 25 mM Tricine/KOH (pH 7.8), 2 mM $MgCl_2$ and 2 mM EDTA. All reactions were run under nitrogen in sealed ampules at roughly 20 °C. The reaction mixture was the same as the chloroplast suspension medium except it also contained 2 mM pyruvate (potassium salt). Samples were prepared and assays for phosphoenolpyruvate performed as described above.

RESULTS AND DISCUSSION

Effect of pH and substrates on the distribution of phosphoenolpyruvate across the chloroplast envelope

Isolated intact mesophyll chloroplasts of *D. sanguinalis* contain a pool of endogenous phosphoenolpyruvate which distributes across the envelope. As shown in Fig. 1, in the absence of external substrates (control) approx. 75 % of the total phosphoenolpyruvate was found in the supernatant with 25 % retained in the stroma. In this experiment, the chloroplasts were separated from the medium by silicone oil centrifugal filtration after a 10 s incubation. That the appearance of phosphoenolpyruvate in the supernatant was not due to breakage of the chloroplasts as they were centrifuged through the silicone oil is suggested by control experiments in which it was determined that chloroplasts retain both a stromal enzyme (NADP-malate dehydrogenase) and internal adenylates (Huber, S. C., unpublished). Rather, the phosphoenolpyruvate may be distributing according to the pH gradient across the envelope. In the experiment of Fig. 1 (control), the pH of the stroma (determined by the distribution of $H^{14}CO_3^-$ [9]) was found to be 8.2 and the pH of the medium was 7.8. The concentration of phosphoenolpyruvate in the supernatant was 18.4 μM and the concentration of phosphoenolpyruvate in the stroma was calculated to be 375 μM. If phosphoenolpyruvate (PEP) was distributing according to a pH gradient, the ratio would be given by [10]:

$$\log \frac{[\mathrm{PEP}]_{\mathrm{internal}}}{[\mathrm{PEP}]_{\mathrm{external}}} = n(\mathrm{pH_{in}} - \mathrm{pH_{out}})$$

Assuming $n = 3$ for phosphoenolpyruvate, the ratio (internal/external) would be

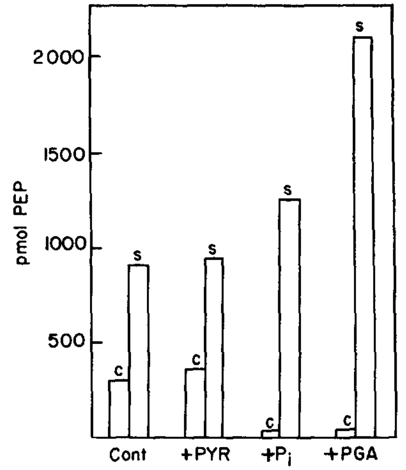

Fig. 1. The effect of substrates on the efflux of phosphoenolpyruvate from intact mesophyll chloroplasts of *D. sanguinalis*. Chloroplasts were incubated in 50 μl of a mixture containing 0.3 M sorbitol, 50 mM Tricine/KOH (pH 7.8), 2 mM $MgCl_2$, 2 mM EDTA and, as indicated, 1 mM pyruvate (PYR), 1 mM P_i or 1 mM 3-phosphoglycerate (PGA). After 10 s of incubation at 20 °C in the dark, the chloroplasts were separated from the medium by silicone oil centrifugal filtration and the supernatant (S) and chloroplast pellets (c) were assayed for phosphoenolpyruvate as described in Materials and Methods. Each reaction mixture contained 12 μg chlorophyll which corresponds to a stromal space of 0.80 μl [5].

expected to be roughly 16 under the above conditions. The observed ratio was 20 (375/18), which suggests that the observed distribution of phosphoenolpyruvate may be dependent on the pH gradient across the envelope.

Pyruvate did not affect the observed distribution of phosphoenolpyruvate, whereas external P_i or phosphoglycerate reduced the phosphoenolpyruvate in the stroma to very low levels. This may indicate that P_i, phosphoenolpyruvate and phosphoglycerate share a common translocator (the phosphate translocator) while pyruvate is not a substrate of the carrier. The total amount of phosphoenolpyruvate (supernatant + stroma) was constant at roughly 1250 pmol for the control and in the presence of pyruvate or P_i. In contrast, the amount of phosphoenolpyruvate appearing in the supernatant in the presence of phosphoglycerate was higher than expected. This may be due to some conversion of phosphoglycerate to phosphoenolpyruvate within the chloroplast by the enzymes phosphoglycerate mutase and enolase [3].

Uptake of $^{32}P_i$

Kinetics of $^{32}P_i$ uptake could be resolved at 18 °C at low substrate concentrations. Fig. 2 shows that P_i uptake (10 μM $^{32}P_i$ external) appears to follow a first-order reaction. A semilogarithmic plot of the data (Fig. 2, insert) is linear and from the slope of the line a rate constant of 3.0 min^{-1} can be calculated. The line does not pass through the origin but rather intercepts the ordinate at roughly 2 s, which corresponds to the time lag required to terminate the uptake reaction by centrifugation through the silicone oil. Similar time lags have been observed for [^{14}C]-pyruvate uptake as well (Huber, S. C., unpublished). First-order kinetics have been shown for

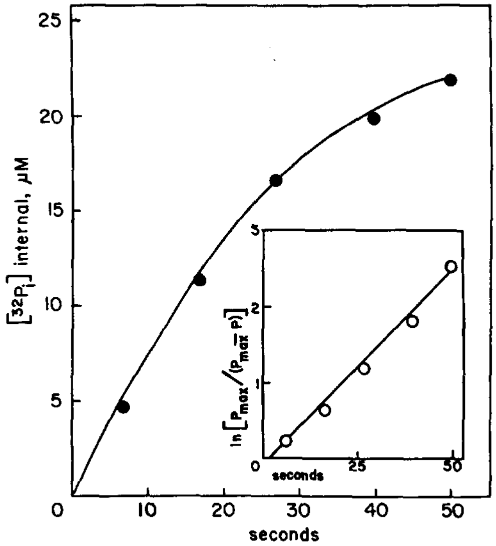

Fig. 2. Time dependence of $^{32}P_i$ uptake by intact chloroplasts in the dark at 18 °C. Chloroplasts were incubated in a medium containing 0.3 M sorbitol, 50 mM Tricine/KOH (pH 7.8), 10 mM K_2SO_4 and 10 μM $^{32}P_i$. At the times indicated, uptake was terminated by silicone oil centrifugal filtration. The insert shows a semilogarithmic plot of the data according to the relation $\ln[P_{max}/(P_{max}-P)] = kt$, where P_{max} is the steady-state internal concentration and P is the internal concentration at time t. The first-order constant calculated from the slope of the line is 3.0 min^{-1}.

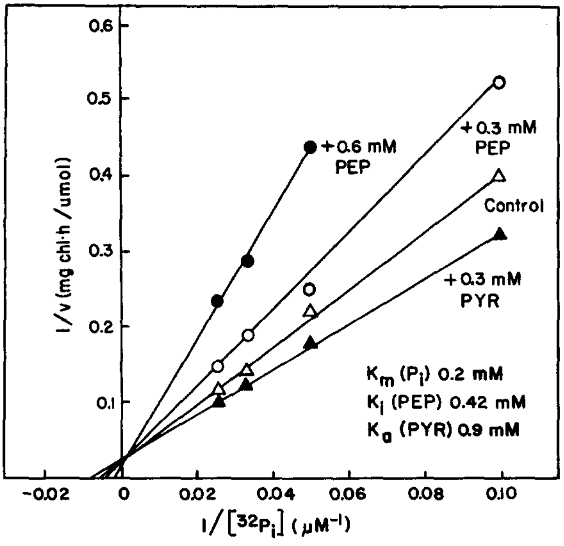

Fig. 3. Effect of P_i concentration on the velocity of $^{32}P_i$ uptake by chloroplasts in the dark at 18 °C. Conditions were as described in Materials and Methods.

the adenine nucleotide [11], succinate [12] and citrate [13] transfer across the mitochondrial membrane.

The effect of P_i concentration on the initial velocity of $^{32}P_i$ uptake is shown in Fig. 3. It should be noted that the experiment of Fig. 3 was performed by necessity at rather low P_i concentrations (10–40 μM) in order to resolve uptake kinetics. At higher concentrations of P_i, initial velocities could not be resolved (data not shown). The data obtained at low substrate concentrations suggests that P_i uptake follows Michaelis-Menten kinetics. Without added effectors (Fig. 3, control), an extrapolated V of 50 μmol/mg chlorophyll per h and apparent $K_m(P_i)$ of 200 μM are obtained at 18 °C. Rates of pyruvate- and P_i-dependent CO_2 fixation by mesophyll protoplast extracts of *D. sanguinalis* (rate of CO_2 fixation = rate of P_i uptake) at 18 °C are in the order of 25–50 μmol/mg chlorophyll per h (Huber, S. C., unpublished) suggesting that the uptake observed in the dark is sufficient to account for the rate of pyruvate metabolism in the light. An apparent $K_m(P_i)$ of 0.2 mM is also consistent with the previously reported low levels of P_i required for saturation of pyruvate metabolism by *D. sanguinalis* mesophyll protoplast extracts [4].

Phosphoenolpyruvate was a competitive inhibitor of the P_i uptake (Fig. 3). The competitive nature of the inhibition suggests that phosphoenolpyruvate and P_i share a common carrier, which is likely an exchange-diffusion translocator. Spinach (C_3) chloroplasts have a phosphate (exchange-diffusion) translocator which transports P_i, 3-phosphoglycerate and dihydroxyacetonphosphate, while phosphoenolpyruvate (PEP) is a rather poor transport metabolite [6]. Inhibition constants (K_i) can be calculated from the data using the following relation for a competitive inhibitor:

$$x\text{-intercept} = \frac{1}{K_m(1+[I]/K_i)}$$

On this basis, the apparent K_i (PEP) of $^{32}P_i$ uptake was 425 μM. It has been shown with the phosphate and dicarboxylate exchange-diffusion carriers of spinach (C_3) chloroplasts [6] and the transport of pyruvate and lactate in erythrocytes [14] that the K_i for a substrate is equivalent to the K_m for that substrate for transport. On this basis, we tentatively conclude that the apparent K_i(PEP) for $^{32}P_i$ uptake approximates the K_m(PEP) for transport.

The apparent $K_m(P_i)$ obtained for C_4 mesophyll chloroplasts is similar to the value reported for spinach (C_3) chloroplasts [15]. However, the K_i(PEP) obtained with the C_4 mesophyll chloroplasts is roughly 10-fold lower than the K_i(PEP) with spinach [15], the latter having a low capacity for phosphoenolpyruvate transport. Hence, the phosphate translocator of the C_4 mesophyll chloroplast appears to be different than that of the C_3 mesophyll chloroplast in terms of substrate specificity. This is quite logical, since phosphoenolpyruvate is proposed to be a photosynthetic intermediate in C_4 plants (requiring transport across the mesophyll envelope) while it is not in the C_3 chloroplast.

In contrast to phosphoenolpyruvate, pyruvate stimulated the rate of $^{32}P_i$ uptake (Fig. 3), which suggests that pyruvate and P_i do not share a common carrier. Pyruvate uptake by C_4 mesophyll chloroplasts has been shown to be carrier mediated and has been suggested to occur as permeation of the anion via an electrogenic carrier [5].

The effect of P_i concentration on the steady-state concentration of P_i in the

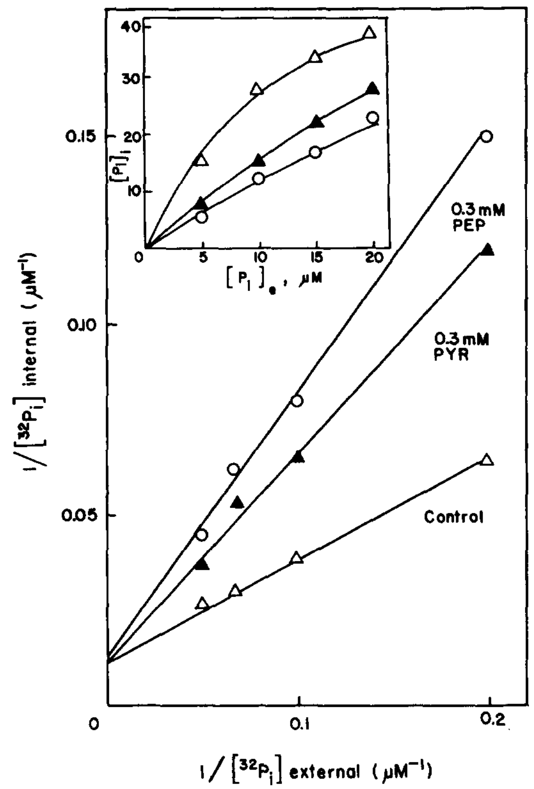

Fig. 4. Effect of P_i concentration (5–20 μM) on the steady-state internal concentration of $^{32}P_i$. Chloroplasts were incubated for 4 min in the dark at 18 °C as described in Materials and Methods without added effectors (control) or in the presence of 0.3 mM phosphoenolpyruvate (PEP) or pyruvate (PYR). The insert shows a standard plot of internal (i) versus external (e) concentrations for the data given in the double-reciprocal plot.

stroma is shown in double-reciprocal form in Fig. 4. For convenience the insert shows a standard plot of internal versus external concentrations. At low external concentrations, P_i was accumulated in the stroma, which would be expected for an exchange-diffusion carrier [6, 7]. As the external concentration was increased, the internal concentration showed saturation of uptake (Fig. 4, insert) such that a double-reciprocal plot of the data yielded a straight line (Fig. 4). As shown, both phosphoenolpyruvate and pyruvate (present at 0.3 mM) reduced the steady-state concentration of P_i in the stroma. This would be expected for phosphoenolpyruvate, since phosphoenolpyruvate and P_i share a common translocator (Fig. 3). The reduction by pyruvate cannot be explained on the same basis since pyruvate and P_i do not share a common carrier (Fig. 3 and ref. 5). However, this result is consistent with the previous demonstration [5] that addition of unlabeled P_i reduces the internal concentration of [^{14}C] pyruvate.

Phosphate requirement for phosphoenolpyruvate formation by chloroplasts in the light

One of the main functions of the C_4 mesophyll chloroplast appears to be the

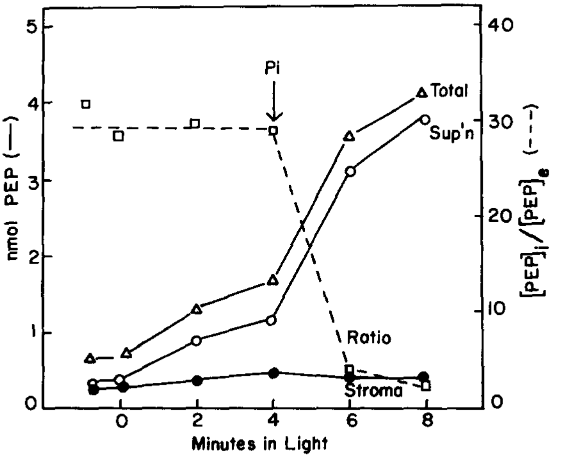

Fig. 5. Effect of Pi on the rate of formation and levels of phosphoenolpyruvate in intact chloroplasts in the light at 20 °C. Chloroplasts were incubated in a CO_2-free mixture as described in Materials and Methods. After 4 min in the light in the presence of 2 mM pyruvate, 0.1 mM Pi was injected and at the times indicated, the chloroplasts were separated from the incubation medium by centrifugation through silicone oil. Phosphoenolpyruvate was assayed as described in Materials and Methods.

formation of phosphoenolpyruvate from pyruvate. This reaction is catalyzed by the enzyme pyruvate, orthophosphate dikinase and requires ATP and P_i. The P_i incorporated into phosphoenolpyruvate has been shown to be derived from the β-phosphate group of ATP [16]. Since the phosphoenolpyruvate formed is subsequently carboxylated in the cytosol of the mesophyll cell, continued formation of phosphoenolpyruvate would require continual uptake of P_i (released in the cytosol by the carboxylase reaction). Pyruvate-dependent CO_2 fixation by mesophyll protoplast extracts of *D. sanguinalis* requires exogenous P_i for maximal rates of CO_2 fixation [4]. If the cytoplasmic fraction (which contains phosphoenolpyruvate carboxylase) is passed through a Sephadex column to remove low molecular weight salts (i.e. P_i) the rate of CO_2 fixation can be increased 15-fold by addition of 1 mM P_i (Huber, S. C. and Edwards, G. E., unpublished). These experiments indicate that the C_4 mesophyll chloroplast is a phosphate-consuming organelle.

The effect of exogenous P_i on the rate of phosphoenolpyruvate formation and subsequent transport is shown in Fig. 5. When C_4 mesophyll chloroplasts are incubated in the light with pyruvate but without P_i, the rate of phosphoenolpyruvate formation was low and the concentration ratio (internal/external) for phosphoenolpyruvate was roughly 30. The addition of 0.1 mM P_i after 4 min resulted in a stimulation of the rate of phosphoenolpyruvate formation and also decreased the concentration ratio (internal/external) for phosphoenolpyruvate from 30 to roughly 2. Whether external P_i is absolutely required for phosphoenolpyruvate formation over a short time period would depend on the concentration of P_i in the stroma. The concentration of P_i in the stroma in the absence of pyruvate was found to be less than 2 mM (Huber, S. C. and Edwards, G. E., unpublished) which is in the order of the reported $K_m(P_i)$ for pyruvate, orthophosphate dikinase (1.5 mM [17]). The results do indicate, however, that external P_i is important in the efflux of photosynthetically generated phosphoenolpyruvate from the stroma.

CONCLUDING REMARKS

This is the first report which directly demonstrates activity of a phosphate translocator in isolated mesophyll chloroplasts of a C_4 plant. In this communication, evidence is presented that P_i and phosphoenolpyruvate compete for uptake via a common carrier, which is probably analogous to the phosphate translocator of spinach (C_3) mesophyll chloroplasts which exchanges internal for external phosphate compounds, although phosphoenolpyruvate is not readily transported [6, 15]. The apparent $K_m(P_i)$ was 0.2 mM and the K_i(PEP), which can be taken as an approximation of the K_m(PEP), was 0.42 mM (Fig. 3). The kinetic constants derived from uptake studies in the dark are comparable to those which are required during metabolism (phosphoenolpyruvate formation) of the chloroplasts in the light [4].

Phosphoenolpyruvate formation by C_4 mesophyll chloroplasts in the light involves the influx of pyruvate and P_i and the efflux of phosphoenolpyruvate from the stroma (Fig. 5 and refs. 2 and 4). In order to operate, these transport processes must be overall electroneutral. It has been suggested that pyruvate uptake occurs via an electrogenic carrier [5], while the present communication provides evidence for an exchange of P_i and phosphoenolpyruvate. This report does not consider the charge balance involved in the exchange of phosphoenolpyruvate $^{3-}$ for P_i^{2-} (charges assigned on the basis of the predominant species at physiological pH) although it is interesting to speculate that the exchange may be electrogenic since pyruvate uptake has been shown to occur via an electrogenic carrier [5].

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