Biochimica et Biophysica Acta, 4 2 (1977) 583–602

BBA 47416

TRANSPORT IN C_4 MESOPHYLL CHLOROPLASTS
CHARACTERIZATION OF THE PYRUVATE CARRIER

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(Received April 12th, 1977)

SUMMARY

1. Evidence is presented for high rates of carrier-mediated uptake of pyruvate into the stroma of intact mesophyll chloroplasts of the C_4 plant *Digitaria sanguinalis*, but not the chloroplasts of the C_3 plant *Spinacea oleracea*. Uptake of pyruvate in the dark with the C_4 mesophyll chloroplasts was followed using two techniques: uptake of [^{14}C]pyruvate as determined by silicon oil centrifugal filtration and uptake as indicated by absorbance changes at 535 nm (shrinkage/swelling) after addition of 0.1 M pyruvate salts.

2. Uptake of the pyruvate anion by an electrogenic carrier is suggested to be the major mode of transport. Chloroplast swelling was observed in potassium pyruvate plus valinomycin and uptake of [^{14}C]pyruvate was inhibited by membrane-permeant anions. Valinomycin reduced uptake in the absence of external potassium and the inhibition could be reversed by addition of external potassium.

3. Uptake of pyruvic acid (or a pyruvate $^-$/OH^- antiport) is ruled unlikely since [^{14}C]pyruvate uptake was relatively independent of the pH gradient across the envelope and addition of pyruvate to chloroplasts did not result in an alkalization of the medium. The low rate of swelling observed in ammonium pyruvate may be due to non-mediated permeation of pyruvic acid, which is possible only at high pyruvate concentrations.

4. The concentration of pyruvate in the stroma increased with external concentration over the range tested (up to 40 mM) but the concentration ratio (internal/external) was always less than one. The steady-state concentration of [^{14}C]pyruvate in the stroma was dependent on the ionic strength of the medium, with saturation at roughly $I = 0.04$ M, while accumulation of the membrane-permeant cation tetraphenylmethylphosphonium decreased with increasing ionic strength. This suggests that ionic strength modifies a membrane potential (inside negative) across the envelope and that pyruvate uptake responds to the magnitude and direction of that potential (-80 mV at low ionic strength).

Abbreviations: C_3 plant, plant having the Calvin-Benson pathway of photosynthesis; C_4 plant, plant having the C-4 dicarboxylic acid pathway of photosynthesis.

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5. Chloride and inorganic phosphate were potent inhibitors of [^{14}C]pyruvate uptake. Of the sulfhydryl reagents tested, *N*-ethylmaleimide was not inhibitory while mersalyl completely blocked [^{14}C]pyruvate uptake and swelling in potassium pyruvate plus valinomycin. Pyruvate uptake, as measured by valinomycin induced swelling in potassium pyruvate, was highly temperature sensitive, with an energy of activation of 39 kcal/mol above 9 °C.

6. Phenylpyruvate, α-ketoisovalerate, α-ketoisocaproate, α-cyano-4-hydroxycinnamic acid and α-cyanocinnamic acid inhibited [^{14}C]pyruvate but not [^{14}C]-acetate uptake in the dark and also reduced pyruvate metabolism by the chloroplasts in the light.

INTRODUCTION

Transport of pyruvate, a photosynthetic intermediate in plants having the C_4-dicarboxylic acid pathway of photosynthesis, across the mesophyll chloroplast envelope of C_4 plants has been postulated to occur in vivo [1] and has been indirectly demonstrated in vitro [2–4]. The requirement for pyruvate transport across the mesophyll chloroplast envelope stems from compartmentation of the enzymes of the carboxylation phase of the C_4-dicarboxylic acid pathway in the mesophyll cell: pyruvate, orthophosphate dikinase is chloroplastic while phosphoenolpyruvate carboxylase is in the cytosol [4–6]. Pyruvate from the cytoplasm is thought to be converted in the chloroplast stroma to phosphoenolpyruvate, through pyruvate, orthophosphate dikinase, which is then carboxylated in the cytosol to form oxaloacetate. Transport of pyruvate in, and phosphoenolpyruvate out, of the chloroplast is therefore required. Pyruvate is not thought to be readily transported across the envelopes of spinach (a C_3 plant) chloroplasts [7] whose carbon metabolism differs from the mesophyll chloroplasts of C_4 plants. Since pyruvate is not a photosynthetic intermediate in spinach chloroplasts, there is no need for such transport.

The present communication reports experiments designed to directly study the transport of pyruvate using intact mesophyll chloroplasts isolated from the C_4 plant *Digitaria sanguinalis*. The need for such a study has been recently documented [7, 8] as there is currently no evidence for the metabolite transport that is proposed to occur in C_4 plants. Evidence is presented that pyruvate uptake is carrier mediated and that the pyruvate anion can enter the chloroplasts while uptake of pyruvic acid (or the equivalent, pyruvate $^-$/OH^- antiport) is relatively inert. Various inhibitors of pyruvate transport in mitochondria are shown to be effective inhibitors of [^{14}C]pyruvate uptake in the dark and pyruvate metabolism (phosphoenolpyruvate formation) in the light by C_4 mesophyll chloroplasts. All of the uptake studies reported here were performed in the dark, so that transport could be studied in the absence of pyruvate metabolism to phosphoenolpyruvate.

EXPERIMENTAL

Materials. Chemicals: Except as indicated, all reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). α-Cyanocinnamic acid and its derivatives were kindly supplied by Dr. A. P. Halestrap; tributyltin by Dr. H. A. Lardy; silicon oil type

F-50 by General Electric, Silicon Products Division; and tetra[^{3}H]phenylmethylphosphonium (54 Ci/mol) by Dr. J. Adler. [^{14}C]Pyruvate (135 Ci/mol), [^{3}H]leucine (80 Ci/mmol) and [^{14}C]acetate (54 Ci/mol) were obtained from New England Nuclear.

Chloroplasts: Mesophyll chloroplasts of *D. sanguinalis* (90–98 % intact on the basis of enzyme retention and ferricyanide exclusion) were obtained from mesophyll protoplasts as previously described [3]. Protoplasts were isolated as previously described [9] except they were purified from cellular debris by centrifugation (400 g, 6–8 min) at room temperature over a cushion of Sigma Ficoll type F-P, similar to the method of Larkin [10]. Chloroplasts were prepared by mechanically rupturing purified mesophyll protoplasts by passage through a 20 μm nylon net [3]. The breaking medium and chloroplast resuspension medium usually contained 0.3 M sorbitol and 25 mM Tricine/KOH (pH 7.7) or 25 mM Tricine/Tris (pH 7.7), as indicated. Spinach (C_3) chloroplasts were mechanically isolated as described by Lilley et al. [11].

Methods. Silicon oil centrifugal filtration: Chloroplasts were rapidly separated from the incubation medium by centrifugation through a layer of silicon oil (General Electric type F-50) into a bottom layer of 20 μl of 10 % $HC10_4$ as previously described for spinach chloroplasts [12]. The 70 μl incubation medium normally contained 0.3 M sorbitol, 25 mM Tricine/KOH (pH 7.7), 15 mM K_2SO_4 and 26 μM [^{14}C]pyruvate (135 Ci/mol). Reactions were typically initiated by the addition of chloroplasts (5–10 μg chlorophyll) and terminated by centrifugation. After centrifugation, 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 placed in scintillation fluid to determine dpm of label 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 volumes were determined by uptake of ^3H_2O and [^{14}C]sucrose as previously described [12]. The pH of the stroma was determined by the distribution of 1 mM $NaH^{14}CO_3$ (12 Ci/mol) according to the relation [13]:

$$\log \frac{[H^{14}CO_3]_{int}}{[H^{14}CO_3]_{ext}} = n\Delta pH$$

One-dimensional paper (Whatmann 1 MM) chromatography (butanol/acetic acid/H_2O, 4 : 1 : 1, v/v for 4 h) of the [^{14}C]pyruvate in the pellet revealed only a single peak which co-chromatographed with the stock [^{14}C]pyruvate and cold authentic pyruvic acid (r_F = 0.42). If chloroplasts were exposed to light during label uptake, however, a second peak of radioactivity was observed which co-chromatographed with phosphoenolpyruvate (r_F = 0.17). All uptake experiments were performed in the dark at roughly 20 °C.

Chloroplast swelling studies: Chloroplasts, suspended in 0.25 M sorbitol and 20 mM Tricine/KOH (pH 7.7), were added to a 1.2 ml cuvette containing 1.1 ml of the same mixture to give a final chlorophyll concentration of 20–30 μg/ml. Absorbance was followed at 535 nm with a Beckman Acta III double-beam spectrophotometer. Solutes were added from 2 M stocks to give a final concentration of 0.1 M. Simultaneous with the addition of solute, a commensurate amount of chlorophyll was added to maintain a constant concentration of chlorophyll.

Assay for pyruvate metabolism by chloroplasts: Mesophyll protoplast extracts were prepared and $^{14}CO_2$ fixation assays performed as previously described [37]. The

basic reaction mixture for $^{14}CO_2$ fixation contained 0.3 M sorbitol, 2 mM $MgCl_2$, 1 mM K_2HPO_4, 50 mM Tricine/KOH (pH 7.7) 2 mM pyruvate and 2 mM $NaH^{14}CO_3$. Other additions were as indicated in the text. All rates were calculated from the linear phase of $^{14}CO_2$ fixation and are expressed as μmol $^{14}CO_2$ fixed/mg chlorophyll per h.

RESULTS AND DISCUSSION

Osmotic response of chloroplasts to pyruvate salts

The osmotic response of intact chloroplasts to different pyruvate salts is shown in Fig. 1. Following the addition of 100 mM salt there is a rapid absorbance increase which indicates chloroplast shrinkage due to the increase in osmolarity of the medium.

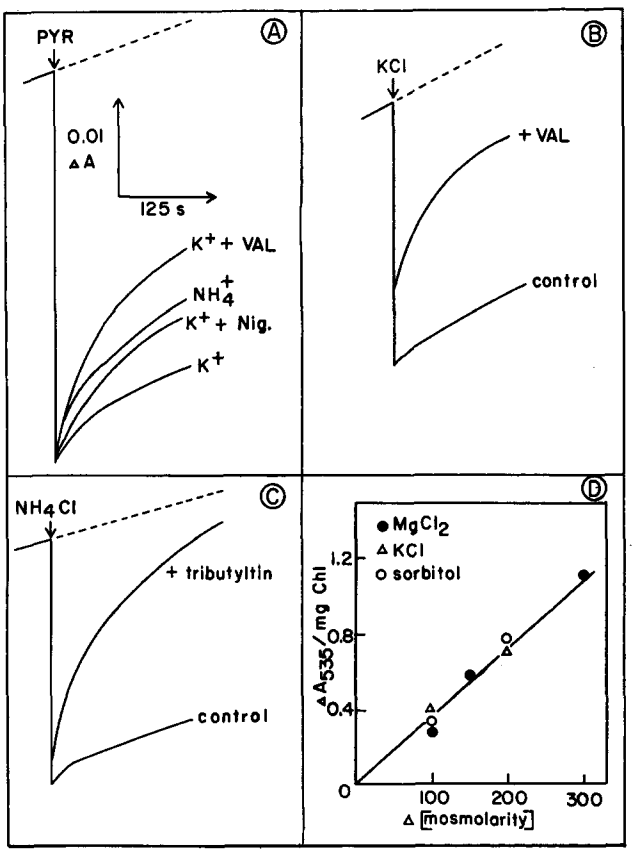

Fig. 1. Kinetics of the changes in absorbance at 535 nm induced by the addition of (A) pyruvate salts, (B) KCl and (C) NH_4Cl. The plot in D shows the relation of the observed change in absorbance to change in medium osmolarity. Substrates were added as indicated by the arrows to give a final concentration of 0.1 M. The dashed line indicates the absorbance change if an equal amount of reaction mixture was added instead of substrate. Where used, 2 μM valinomycin (VAL), 1 μM nigericin (Nig) and 10 μM tributyltin were present from the start of the experiment. A negative deflection indicates an absorbance increase.

If the substrate can enter the chloroplast, the initial absorbance increase will be reversed, due to chloroplast swelling [14]. As shown in Fig. 1A, the addition of 100 mM potassium pyruvate resulted in a large absorbance increase which was only partially reversed. In the presence of valinomycin, however, the reversal was rapid and substantial. Chloroplasts will swell only if both the cation and anion can penetrate the envelope. The finding that swelling in potassium pyruvate required valinomycin indicates that the pyruvate anion is permeable while potassium permeability requires valinomycin. Similar evidence by Heber et al. [14] indicated that the glycerate anion was permeable to spinach chloroplast envelopes. Pyruvate anion permeation is apparently species specific, as there was little swelling of spinach (C_3) chloroplasts in potassium pyruvate ($\pm$ valinomycin; data not shown).

Some swelling in potassium pyruvate in the absence of valinomycin may indicate partial permeability to potassium or it may reflect permeation of pyruvic acid (100 mM potassium pyruvate at pH 7.7 is in equilibrium with roughly 1.0 μM pyruvic acid). There was significant chloroplast swelling in ammonium pyruvate (Fig. 1A) which also suggests that pyruvate can enter the chloroplast as the acid (non-mediated diffusion) or alternatively, as a pyruvate $^-/OH^-$ antiport (carrier mediated). Although mechanistically different, both would be facilitated by the presence of ammonium in the medium. An important observation is that swelling in ammonium pyruvate was significant and reproducible, but limited in extent. This suggests that if pyruvate is entering by one of the mechanisms listed above, the transport is limited by some factor other than internal OH^- (provided by diffusion of NH_3 into the stroma where it forms $NH_4^+ + OH^-$).

Alternatively, the observed swelling in ammonium pyruvate may reflect non-mediated diffusion of pyruvic acid through the envelope. Regardless of the mechanism, additional evidence for uptake of pyruvic acid is the enhanced swelling in potassium pyruvate plus nigericin (Fig. 1A). Presumably nigericin facilitates swelling by removing protons, taken up with the pyruvate, from the stroma in exchange for external potassium.

As a control, swelling studies were performed with the potassium and ammonium salts of chloride. As shown in Fig. 1B, there was essentially no chloroplast swelling in response to 100 mM KCl, which indicates that the envelope is impermeable to either potassium or chloride or both. In the presence of valinomycin, considerable swelling was observed in KCl (Fig. 1B). This suggests that the envelope is permeable to chloride but impermeable to potassium, such that in the presence of valinomycin both the cation and the anion can enter the stroma and swelling is observed. Permeability of spinach chloroplast envelopes to chloride has also been reported [14]. There was also no swelling in NH_4Cl (Fig. 1C). Swelling in NH_4Cl would require either uptake of HCl or a Cl^-/OH^- antiport across the envelope. Trialkyltin compounds are known to catalyze a linked exchange of OH^- and Cl^- across biological membranes [15]. As a result of this activity, rapid swelling of chloroplasts in NH_4Cl was induced by 10 μM tributyltin (Fig. 1C).

The osmotic response of chloroplasts, as indicated by an increase in absorbance at 535 nm, was linearly related to the change in osmolarity of the medium (Fig. 1D). At a constant concentration, the change in absorbance induced by $MgCl_2$, KCl and sorbitol was in the expected ratio 3 : 2 : 1. The absorbance increase induced by these compounds was concentration dependent and is thought to result by increased light

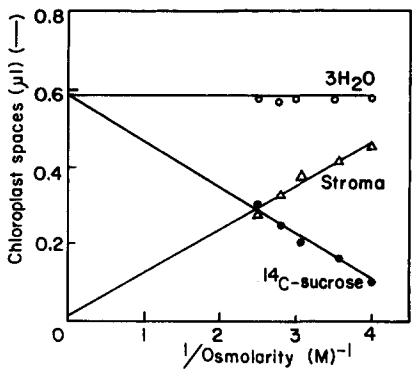

Fig. 2. Effect of osmolarity of the incubation medium on measured chloroplast spaces. ^3H_2O and [^{14}C]sucrose spaces were calculated as described in Methods; the stromal space is assumed to be the difference. The reaction mixture contained 20 mM Tricine/KOH (pH 7.7) and sorbitol to give the indicated osmolarity, which was calculated to include the contribution from the buffer. Each reaction contained 6 μg chlorophyll.

scattering of the particles as they shrink. The fact that KCl and sorbitol are impermeable and cause particle shrinkage was determined by direct measurement of chloroplast volumes. The results of such an experiment where the concentration of sorbitol was varied is shown in Fig. 2. As described in Methods, the total chloroplast volume was determined as the ^3H_2O space and the intermembrane space as that occupied by [^{14}C]sucrose; the stroma is assumed to be sucrose impermeable and was taken as the difference. As shown in Fig. 2, the total chloroplast volume (^3H_2O space) remained constant over the sorbitol concentration range tested (0.23–0.40 M) while the intermembrane space ([^{14}C]sucrose) increased linearly with the inverse of osmolarity. This indicates that sorbitol is impermeable and furthermore that the permeability barrier to sorbitol is the inner, rather than the outer, membrane of the chloroplast envelope.

When the data are extrapolated to infinite osmolarity, the stromal space decreases to near zero and the entire chloroplast volume consists of intermembrane space. Comparable results were obtained when the concentration of KCl was varied (data not shown). Similar plots of organelle spaces versus inverse osmolarity have been obtained for mitochondria [16]. The data support the earlier proposal from studies with spinach chloroplasts [12] that the inner membrane of the chloroplast is the permeability barrier to various solutes and confirm the notion that absorbance changes at 535 nm reflect changes in particle size. An interesting comparison between C_3 and C_4 mesophyll chloroplasts is that the stromal space of the C_4 is roughly 2-fold larger per mg chlorophyll; at a sorbitol concentration of 0.3 M, the stromal space in the C_4 averaged 60 ± 5 μl/mg chlorophyll (average of ten replications) compared to roughly 25 μl/mg chlorophyll in spinach (C_3) chloroplasts [12].

Uptake of [^{14}C]*pyruvate into the stroma.* The osmotic response experiments reported above detect only net transport as metabolite uptake via an exchange reaction will not alter the internal osmolarity. In order to detect uptake via any mechanism, uptake of [^{14}C]pyruvate was followed using the silicon oil centrifugal filtration technique. The dependence of the internal concentration of pyruvate and leucine on external concentration is shown in Fig. 3. The internal concentration of both substrates continued to increase with external concentration up to 40 mM. With leucine

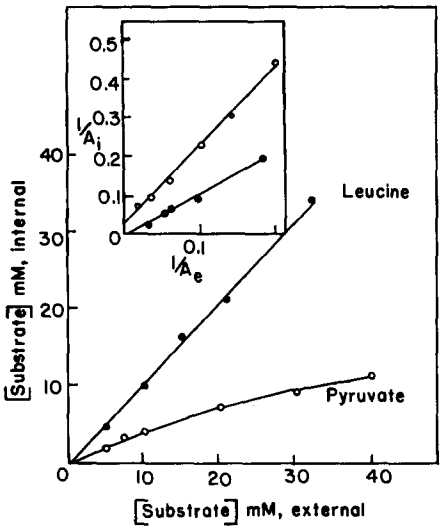

Fig. 3. Internal concentration as a function of external concentration for [^{14}C]pyruvate and [^{3}H]-leucine. Uptake was determined after a 4 min incubation at room temperature in the dark. The basic reaction mixture contained 0.3 sorbitol, 20 mM Tricine/KOH (pH 7.7) and K_2SO_4 to maintain an ionic strength of 0.04 M. The insert shows a double-reciprocal plot of internal (A_i) versus external (A_e) concentration to determine maximal capacity (A_m).

(zero net charge at pH 7) the steady-state internal concentration was roughly equal to the external concentration whereas with pyruvate (−1 charge) there was considerable uptake but the ratio of internal to external concentration at steady state was always less than unity and decreased with increasing external concentration.

Double-reciprocal plots of intra- versus extra-chloroplastic concentrations yields maximal capacity (A_m) levels for the metabolite [17] in analogy to the Lineweaver-Burk plot for enzyme kinetics which yields maximal reaction velocity. The insert of Fig. 3 shows such a plot for leucine and pyruvate. Both plots are linear but the extrapolated A_m (abscissa intercept) for leucine is infinity whereas there is a finite intercept for pyruvate which corresponds to 40 mM internal at infinite external pyruvate. Among several other experiments, the value of A_m (pyruvate) varied between 40 and 80 mM internal.

Metabolites that are transported into spinach chloroplasts by exchange reactions appear to accumulate in the stroma when the external concentration is low [12]. Glycerate, however, has been suggested to be a permeant anoin that is not taken up by a strictly coupled exchange reaction. Similar to the data for pyruvate, the concentration ratio (internal/external) for glycerate was less than one and decreased with increasing concentration [14].

Characteristics of [^{14}C]pyruvate uptake

The kinetics of [^{14}C]pyruvate uptake were followed at room temperature at low external concentrations (10–60 μM). As shown in Fig. 4, at low substrate levels, uptake was complete after roughly 40 s and initial velocities could be determined on the basis of dpm taken up in 7 s (shortest time that could be accurately controlled). At high substrate concentration, it was difficult to resolve uptake kinetics. Fig. 5 is a plot

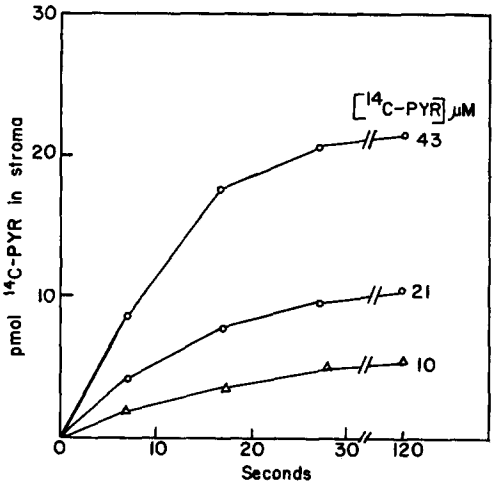

Fig. 4. Kinetics of [^{14}C]pyruvate uptake at room temperature in the dark. Chloroplasts were separated from the incubation mixture at the times indicated by silicon oil centrifugal filtration as described in Methods.

of initial velocities versus substrate concentration from a typical experiment, and the insert shows a double-reciprocal plot of the data. The plot is linear and intercepts the abscissa to yield an extrapolated V of 50 μmol/mg chlorophyll per h and an apparent K_m (pyruvate) of 670 μM. Kinetic constants derived from such measurements made at substrate levels much less than the K_m are subject to some error; from several experiments values for the V ranged from 50 to 100 μmol/mg chlorophyll per h and the apparent K_m (pyruvate) from 0.6 to 1.0 mM. The kinetic constants so determined are of the same order of magnitude as those obtained for pyruvate metabolism to phosphoenolpyruvate (see Methods for details of assay used) by chloroplasts in the light at 20 °C: V, 50–70 μmol/mg chlorophyll per h and apparent K_m (pyruvate) 0.9–1.2 mM

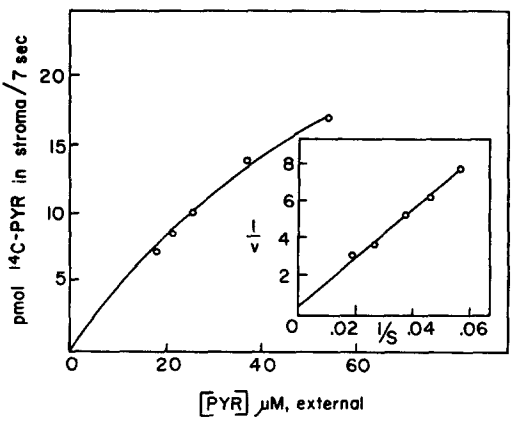

Fig. 5. Velocity of [^{14}C]pyruvate uptake as a function of substrate concentration. Uptake was initiated by the addition of chloroplasts and terminated after 7 s by centrifugation through silicon oil. For details, see Methods. The insert shows a double-reciprocal plot of the data.

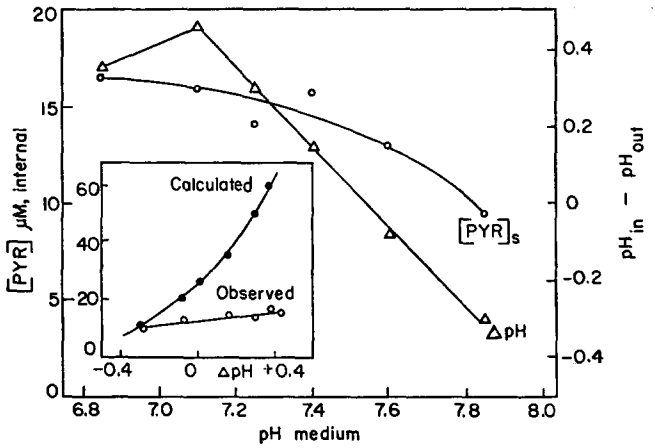

Fig. 6. Effect of external pH on [^{14}C]pyruvate uptake and ΔpH across the envelope. The concentration of [^{14}C]pyruvate in the stroma was determined after a 4 min incubation of the chloroplasts in the dark with 26 μM [^{14}C]pyruvate. The pH of the stroma (pH_s) and pH gradient (ΔpH) across the envelope was determined by the distribution of $H^{14}CO_3^-$. The basic reaction mixture contained 0.3 M sorbitol, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/Tris (pH as shown) and 14 mM $MnSO_4$.

(Huber, S. C. and Edwards, G. E., unpublished). It appears that the observed transport in the dark can account for the transport required in the light during metabolism of pyruvate.

Effect of pH on [^{14}C]pyruvate uptake

To further decide whether pyruvate can enter the chloroplast as the free acid (or via a pyruvate $^-$/OH^- antiport), the steady-state concentration of pyruvate in the stroma was measured as a function of medium pH. As shown in Fig. 6, varying the pH of the medium from 6.8 to 7.8 varied the pH gradient across the chloroplast envelope. As the pH of the medium varied, the pH gradient across the envelope changed in a linear fashion; i.e. the pH of the stroma remained relatively constant. This suggests that the envelope is relatively impermeable to protons and hydroxyls and is consistent with previous findings for spinach chloroplasts [18] and the blue-green alga *Anacystis nidulans* [19]. By varying the medium pH one unit, the pH gradient (in-out) varied from +0.43 to −0.30 unit. The concentration of pyruvate in the stroma however, was relatively pH independent (Fig. 6) and the concentration ratio internal/external remained less than one over the entire range. If pyruvate uptake was driven by a pH gradient, i.e. if pyruvate enters as the undissociated acid or via a pyruvate $^-$/OH^- antiport, the distribution of pyruvate would be given by:

$$\log \frac{[\text{pyruvate}]_{int}}{[\text{pyruvate}]_{ext}} = n\Delta\text{pH}$$

as has been verified for the transport of several metabolites in mitochondria [17, 20], including pyruvate [21]. This relationship predicts that at ΔpH = 0, the internal and external concentrations are equal and as ΔpH increases, the anion is accumulated in the stroma. The insert of Fig. 6 is a plot of internal concentration versus ΔpH for the

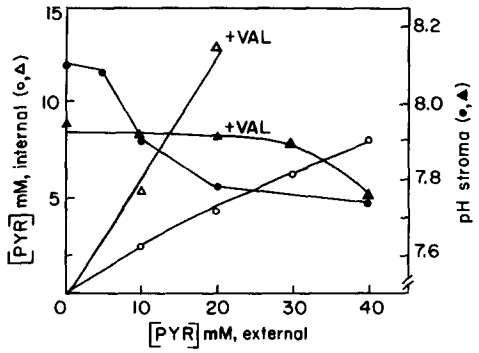

Fig. 7. Effect of external pyruvate on the concentration of internal pyruvate and the pH of the stroma. All measurements were made by silicon oil centrifugation after a 4 min incubation of chloroplasts in the dark. The basic reaction mixture contained 0.3 M sorbitol, 25 mM Tricine/KOH (pH 7.7) and K_2SO_4 to maintain $I = 0.03$. Where indicated, valinomycin (VAL) was added to give a final concentration of 2 μM.

data given and the expected result if pyruvate uptake is driven by ΔpH. As shown, the observed pH dependence is negligible with respect to the calculated case. The experiment of Fig. 6 was performed with 20 mM $MnSO_4$ to provide ionic strength; results were the same if $(NH_4)_2SO_4$ replaced $MnSO_4$ (data not shown).

The relative pH independence of transport (both steady-state internal concentration as well as uptake velocity, data not shown) was highly reproducible and suggest that at low pyruvate concentration (< 1 mM) the uptake driven by ΔpH is minimal. Consistent with this interpretation is the inability to observe a pH change in the external medium upon addition of low levels of pyruvate (< 4 mM, data not shown). Also, if pyruvate accumulated at the expense of a pH gradient, then pyruvate penetration should result in a reduction of the pH gradient between the two spaces. The effect of pyruvate on the pH of the stroma is shown in Fig. 7. In the absence of valinomycin, pyruvate had no effect on the stroma pH below 5 mM, but a reduction was observed as the concentration was increased above 5 mM. This reduction may reflect non-mediated permeation of pyruvic acid. In animal mitochondria which catalyze a pyruvate $^-/OH^-$ exchange, 2 mM pyruvate was observed to decrease the matrix pH by 25 % (from 1.0 to 0.76 unit [21]). In the presence of valinomycin, however, the concentration of pyruvate in the stroma was higher than in the absence of valinomycin and the pH of the stroma was not reduced until the concentration of pyruvate in the medium was quite high (Fig. 7).

Effect of ionic strength and membrane potential on [^{14}C]pyruvate uptake

The uptake of [^{14}C]pyruvate was found to be very dependent on the ionic strength of the medium. Both initial uptake velocity (data not shown) and the steady-state concentration in the stroma (Fig. 8) were stimulated by salts and as shown, the effect was relatively non-specific for a number of different electrolytes. The deviation of points from the curve at low ionic strength (0.02 M, Fig. 8) may be due to the fact that chloride (Fig. 1) and nitrate (data not shown) are permeant anions. The initial rate of pyruvate uptake into the stroma increased with ionic strength up to $I = 0.02$ M (data not shown). In a recent report, Rice and Steck [22] reported that pyruvate flux

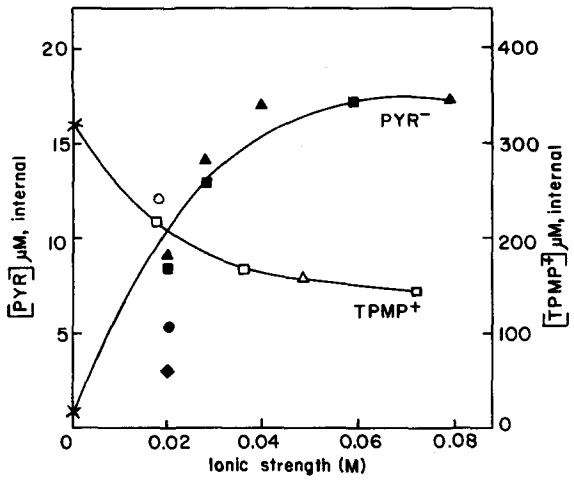

Fig. 8. Effect of ionic strength on the steady-state internal concentrations of [^{14}C]pyruvate (PYR^-, closed symbols) and tetra[^{3}H]phenylmethylphosphonium ($TPMP^+$, open symbols), calculated on the basis of dpm taken up after 4 min. The basic reaction mixture contained 0.3 M sorbitol, 25 mM Tricine/Tris (pH 7.7), 26.4 μM [^{14}C]pyruvate or 13.7 μM tetra[^{3}H]phenylmethylphosphonium and salts as indicated: △, ▲, K_2SO_4; □, ■, $(NH_4)_2SO_4$; ◇, KNO_3; ○, ●, KCl. Ionic strength was calculated as $I = \Sigma Z_i^2 c_i/2$ where Z_i = charge and c_i = molar concentration of the i^{th} species.

into erythrocytes ghosts was very dependent on the ionic strength of the medium, with saturation of transport velocity in the range 0.02–0.04 M. The dependence on ionic strength can be taken as evidence consistent with carrier-mediated transport [27]. Less straightforward is the observation that the steady-state concentration of pyruvate in the stroma was ionic strength dependent (Fig. 8). The observed chloroplast swelling in potassium pyruvate plus valinomycin (Fig. 1) suggested that the envelope is permeable to the pyruvate anion. Assuming this to be true as well in the absence of valinomycin, the fact that the concentration ratio (internal/external) is less than unity (Fig. 3) suggests that uptake of pyruvate may reflect a membrane potential (inside negative). Chloroplasts contain proteins which possess net negative charges at physiological pH. These immobile negative charges give rise to a Donnan potential across the envelope and a Donnan distribution of diffusable ions [23]. A possible explanation of the dependence of uptake on ionic strength is that at high ionic strength, the Donnan potential across the envelope becomes more positive. To test this hypothesis, the effect of ionic strength on uptake of the membrane-permeant cation tetraphenylmethylphosphonium [24] was determined. As shown in Fig. 8, tetraphenylmethylphosphonium was accumulated 10–20-fold in the stroma. The observed accumulation is consistent with a Donnan potential across the envelope (inside negative). As ionic strength was increased, the steady-state concentration of tetraphenylmethylphosphonium in the chloroplast decreased (Fig. 8) indicating that the Donnan potential across the envelope was becoming more positive. Both the increase in pyruvate uptake and decrease in tetraphenylmethylphosphonium uptake plateaued at roughly $I = 0.04$ M.

The magnitude of the Donnan potential can be estimated from the observed distribution of a membrane-permeant ion using the Nernst equation. Potentials

calculated from the distribution of [^{14}C]pyruvate and tetra[^{3}H]phenylmethylphosphonium at very low ionic strength are similar (−80 mV inside negative). However, at high ionic strength (0.04 M), the potentials calculated from the distribution of tetraphenylmethylphosphonium are more negative than those calculated from the distribution of pyruvate (−60 and −15 mV, respectively). The discrepancy in calculated potentials could be explained if some of the pyruvate taken up was bound inside the chloroplast, thereby reducing the activity of the internal pyruvate pool. Alternatively, tetraphenylmethylphosphonium may be taken up into the thylakoid space as well as the stroma, while pyruvate would only enter the stroma. The data indicate, however, that a Donnan potential does exist across the envelope (inside negative) and that pyruvate uptake responds at least qualitatively to the magnitude and direction of that potential. In experiments not reported here, it was determined that tetraphenylmethylphosphonium was very permeable to the chloroplast envelope as kinetics of uptake could not be observed. Uptake studies with tetra[^{3}H]phenylmethylphosphonium were routinely performed at an external concentration of 13 μM; this concentration did not alter the existing membrane potential although as the external concentration was increased (above 20 μM) the potential became more positive (data not shown).

The data presented above suggest that pyruvate uptake reflects a Donnan potential across the envelope. With this in mind, the effect of valinomycin on pyruvate uptake was determined as a function of external potassium concentration. As shown in Fig. 9, valinomycin reduced the equilibrium concentration of pyruvate in the stroma in the absence of external potassium. The inhibition was reversed by exogenous K_2SO_4 whereas KCl resulted in further inhibition. The inhibition of uptake by valinomycin in the absence of external potassium may be interpreted as follows. Chloroplasts normally contain high levels of potassium [25] which can be reduced by valinomycin in the absence of external potassium [23]. The efflux of potassium would be expected to increase the membrane potential (more negative inside) and hence reduce pyruvate uptake. By addition of exogenous potassium the loss of potassium

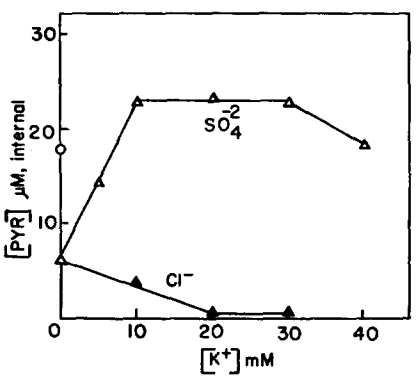

Fig. 9. Effect of external potassium on the steady-state concentration of [^{14}C]pyruvate in the stroma in the presence of 2 μM valinomycin. The basic reaction mixture contained 0.3 M sorbitol, 50 mM Tricine/Tris (pH 7.7) 10 mM $(NH_4)_2SO_4$, 26 μM [^{14}C]pyruvate and potassium salts as indicated. The steady-state internal concentration of [^{14}C]pyruvate in the absence of valinomycin is indicated in the figure by the open circle.

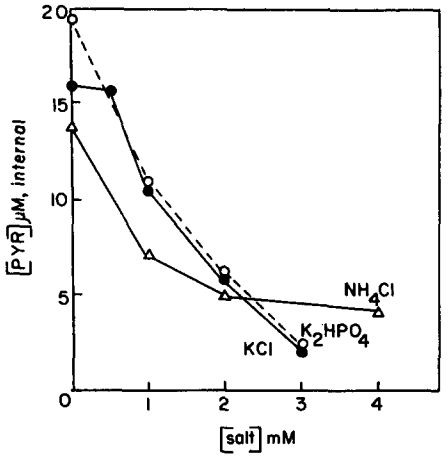

Fig. 10. Effect of chloride and inorganic phosphate on the equilibrium internal concentration of [^{14}C]pyruvate. The basic reaction mixture contained 0.3 M sorbitol, 50 mM Tricine/Tris (pH 7.7), 26 μM [^{14}C]pyruvate and either 15 mM K_2SO_4 (○, △) or 15 mM $(NH_4)_2SO_4$ (●) to give ionic strength. The experiments were performed with different chloroplast preparations on different days.

from the stroma is reduced [23] and inhibition of uptake would be reversed. This effect is observed only when the anion of the potassium salt is not permeable to the envelope. As demonstrated by swelling studies, chloride is a permeant anion (Fig. 1C) whereas sulfate is not (data not shown). Apparently, as KCl is increased in the medium, the loss of internal potassium is reduced, but the uptake of chloride (concentration several mM) swamps the uptake of pyruvate (26 μM).

Inhibition of pyruvate uptake by chloride did not require valinomycin. Both NH_4Cl and KCl as well as potassium phosphate reduced pyruvate uptake with in-

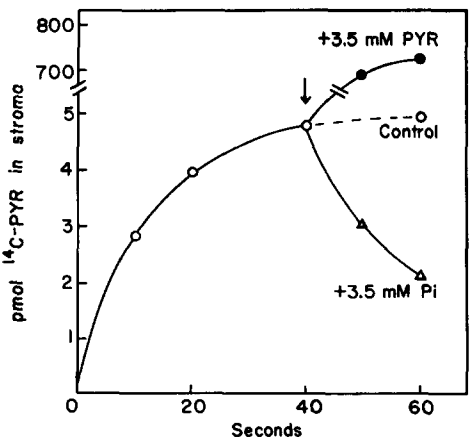

Fig. 11. Effect of addition of unlabelled pyruvate and inorganic phosphate on the concentration of internal [^{14}C]pyruvate. Reactions were initiated by the addition of chloroplasts and were terminated at the times indicated by centrifugation through silicon oil. At 40 s, unlabelled pyruvate or inorganic phosphate was added (in 3 μl) to give a final concentration of 3.5 mM.

creasing concentration (Fig. 10). The observed inhibition may reflect the fact that chloride (Fig. 1) and inorganic phosphate (Huber, S. C. and Edwards, G. E., unpublished) are both permeant anions. A possible explanation of the inhibition observed is as follows. Uptake of pyruvate at low external concentrations proceeds as permeation of the anoin, which distributes according to the membrane potential. Addition of other permeant anions, e.g. chloride or phosphate causes the stroma to become more negative and hence reduces pyruvate uptake.

This theory predicts that addition of a permeant anion should also cause pyruvate efflux from the chloroplast, since uptake of the second anion will increase the membrane potential (more negative inside) and pyruvate will redistribute accordingly. To test this, the experiment of Fig. 11 was performed. After 40 s of [^{14}C] pyruvate uptake, 3.5 mM unlabelled pyruvate or 3.5 mM inorganic phosphate was added and the dpm in the stroma determined with time. As shown, pyruvate uptake reached a steady state after 40 s and addition of unlabelled pyruvate resulted in a slight increase of dpm in the stroma. The concentration ratio (internal/external) remained relatively constant before and after addition of the unlabelled pyruvate (0.34 and 0.40, respectively), implying rapid equilibration of the pyruvate pools in the two compartments. Addition of inorganic phosphate caused a release of [^{14}C]pyruvate from the stroma, consistent with an indirect coupling between the fluxes of permeable anions.

Inhibition of pyruvate transport by sulfhydryl reagents

The effect of *N*-ethylmaleimide and mersalyl on [^{14}C]pyruvate uptake is shown in Fig. 12. Uptake of label was reduced by mersalyl while *N*-ethylmaleimide slightly stimulated the observed uptake. Several carrier-mediated transport systems have been shown to be sulfhydryl reagent sensitive. Uptake of inorganic phosphate by mitochondria [26] and net pyruvate uptake [21] are mersalyl sensitive. Uptake of inorganic

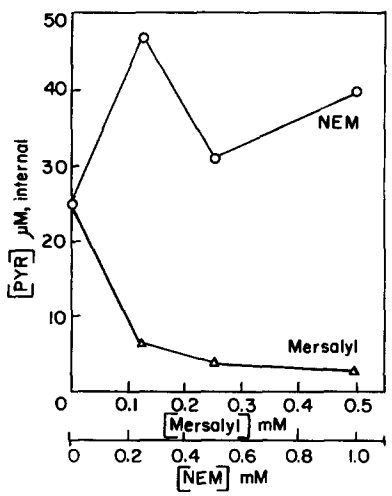

Fig. 12. Effect of mersalyl and *N*-ethylmaleimide (NEM) on the uptake of [^{14}C]pyruvate. Chloroplasts were preincubated in the dark at room temperature with the sulfhydryl reagents for 90 s prior to initiation of uptake by addition of the label. Reactions were terminated after 4 min by centrifugation. Each reaction contained 6 μg of chlorophyll.

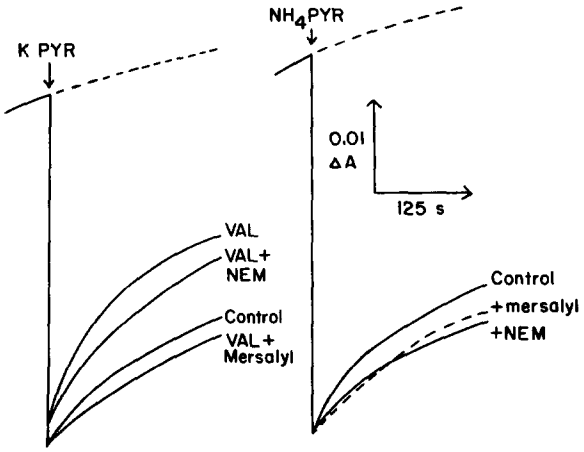

Fig. 13. Effect of mersalyl and *N*-ethylmaleimide (NEM) on chloroplast swelling in (A) potassium pyruvate plus valinomycin and (B) ammonium pyruvate. Conditions were as given in the legend of Fig. 1, except the chloroplasts (20 μg chlorophyll/ml) were preincubated for 90 s with 0.2 mM mersalyl or 0.3 mM *N*-ethylmaleimide prior to addition of substrate.

phosphate by spinach chloroplasts is strongly inhibited by *p*-chloromercuriphenylsulphonic acid [13]. Inhibition of uptake by a sulfhydryl reagent is consistent with, but not a prerequisite for carrier mediation.

The effect of mersalyl and *N*-ethylmaleimide on chloroplast swelling with pyruvate salts was also examined. The effect of these reagents on swelling in potassium pyruvate is shown in Fig. 13A. Mersalyl reduced swelling considerably whereas *N*-ethylmaleimide was without significant effect. In contrast, both reagents resulted in little inhibition of swelling in ammonium pyruvate (Fig. 13B). Since uptake of [^{14}C] pyruvate is mersalyl sensitive and *N*-ethylmaleimide insensitive, it may appear that uptake of the anion is the major mode of penetration in label studies. The low rate of swelling in both cases in the presence of mersalyl may reflect non-mediated diffusion of pyruvic acid, which is possible only at high concentrations of pyruvate.

Effect of temperature on net pyruvate uptake. Initial experiments with [^{14}C] pyruvate uptake indicated that transport was highly temperature sensitive; at 4 °C (condition used for uptake studies with spinach chloroplasts [27]) uptake was very low and difficult to detect as compared to the uptake observed at room temperature (data not shown). This was the experimental basis for conducting all experiments at room temperature. In order to determine more completely the effect of temperature on pyruvate transport, rates of transport as a function of temperature were deduced from the initial rate of chloroplast swelling in potassium pyruvate plus valinomycin. Fig. 14 is an Arrhenius plot of the data obtained. The plot is linear with an apparent break around 9 °C. An activation energy of 39.2 kcal/mol was obtained from the slope of the line. The insert shows a plot of velocity versus temperature. The velocities obtained are clearly rapid enough to account for the maximum rates of pyruvate metabolism by chloroplasts in the light (200–300 μmol/mg chlorophyll per h at 35 °C [3]). Below 9 °C, the V of uptake was very low (< 2 μmol/mg chlorophyll per h) which is consistent with the negligible uptake of [^{14}C]pyruvate observed at 4 °C (data not shown). The results suggest that a membrane phase transition may occur at roughly 9 °C.

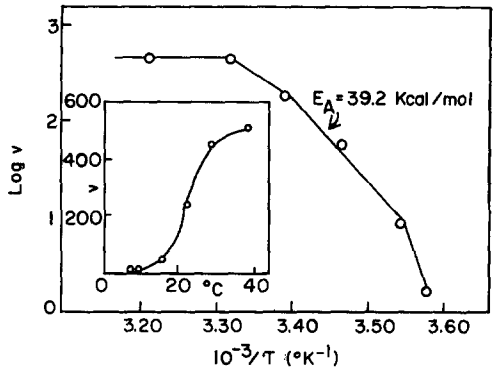

Fig. 14. Effect of temperature on the initial rates of chloroplast swelling in potassium pyruvate plus valinomycin. Conditions as given in the legend of Fig. 1. The reaction mixtures (containing chloroplasts) were preincubated at each temperature for 3 min prior to addition of the potassium pyruvate. Velocity (v) is expressed as μmol/mg chlorophyll per h.

Below the phase transition temperature, valinomycin would not induce potassium permeability and presumably the pyruvate carrier would also be immobile. The ability to observe transport at 4 °C in spinach chloroplasts but not C_4 mesophyll chloroplasts may rest with inherent differences in the lipid composition of the envelope. Poincelot [28] recently reported that the ratio of unsaturated/saturated fatty acids was 3–5-fold lower in maize (C_4) mesophyll envelopes as compared to spinach and sunflower (C_3) envelopes. Increasing amounts of saturated fatty acids are expected to result in a higher temperature of phase transistion [29]. This may explain why transport in C_4 mesophyll chloroplasts is very low at 4 °C while spinach (C_3) chloroplasts continue to transport metabolites at 4 °C (i.e. phase transistions in C_3 envelopes occur at a lower temperature than in C_4).

Inhibition by pyruvate analogues and α-cinnamic acid derivatives of [^{14}C]pyruvate uptake and metabolism

The effect of several pyruvate analogues and α-cinnamic acid derivatives on [^{14}C]pyruvate uptake was determined. Derivatives of α-cinnamic acid have been shown to be specific and potent inhibitors of the mitochondrial pyruvate carrier [30, 31]. The effect of several compounds on [^{14}C]pyruvate and [^{14}C]acetate uptake is given in Table I. As shown, both pyruvate analogues and α-cinnamic acid derivatives resulted in a substantial reduction of the pyruvate taken up whereas those tested had little or no effect on the uptake of acetate, indicating that inhibition was pyruvate specific. Specific inhibition of uptake is further evidence that pyruvate transport is carrier mediated.

The effect of putative pyruvate transport inhibitors on pyruvate metabolism in the light was determined. Fig. 15 is a double-reciprocal plot of the effect of α-cyanocinnamic acid and phenylpyruvate on pyruvate metabolism (phosphoenolpyruvate formation). As shown, both compounds reduced the rate of phosphoenolpyruvate formation; phenylpyruvate was a competitive inhibitor and α-cyanocinnamic acid was a non-competitive inhibitor with respect to pyruvate. The effect of several inhibitors on pyruvate metabolism is summarized in Table II and data for mitochondria are

TABLE I

EFFECT OF PYRUVATE ANALOGUES AND α-CINNAMIC ACID DERIVATIVES ON THE UPTAKE OF [^{14}C]PYRUVATE AND [^{14}C]ACETATE

Uptake was determined as described in Materials and Methods. Labelled pyruvate and acetate were present at a final concentration of 1 mM. Reactions were initiated by the addition of chlorophyll and terminated after 7 s by centrifugation through silicon oil. Results are expressed in nmol taken up/mg chlorophyll per 7 s.

Inhibitor	Concentration (mM)	[^{14}C]Pyruvate	[^{14}C]Acetate
None		15.9	23
Phenylpyruvate	5	8.2	21
	10	4.3	n.d.
α-Ketoisovalerate	5	5.0	n.d.
	10	4.5	n.d.
α-Ketoisocaproate	5	6.0	n.d.
	10	5.2	n.d.
α-Cyanocinnamic acid	1	5.6	24
	2	5.3	n.d.
α-Cyano-4-hydroxy-cinnamic acid	1	4.7	n.d.

n.d., not determined.

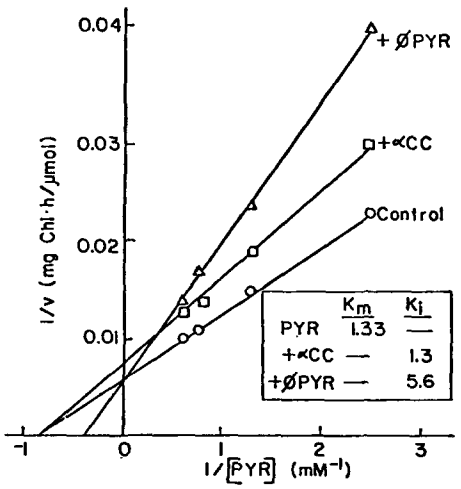

Fig. 15. Inhibition of pyruvate-dependent $^{14}CO_2$ fixation by mesophyll chloroplast preparations of *D. sanguinalis* by 5 mM phenylpyruvate (ϕ PYR) and 0.5 mM α-cyanocinnamic acid (α-C_1). For deails, see Methods.

TABLE II

SUMMARY OF THE EFFECT OF PYRUVATE TRANSPORT INHIBITORS ON PYRUVATE METABOLISM IN THE LIGHT IN COMPARISON TO MITOCHONDRIAL PYRUVATE METABOLISM

Compound	Chloroplast[b]		Mitochondria[a]	
	Type of inhibition[c]	K_i (mM)[d]	Type of inhibition[c]	K_i (mM)[d]
Pyruvate	(app. K_m(pyr) = 1.2 mM)		(app. K_m(pyr) = 0.15 mM)	
Phenylpyruvate	C	5.7	C	1.8
α-Ketoisovalerate	UC	4.8	No inhibition	
α-Ketoisocaproate	UC	5.0	No inhibition	
α-Cyanocinnamic acid	NC	1.2	—	0.0002
α-Cyano-4 hydroxy-cinnamic acid	C	3.1	NC	0.0065
α-Cyano-3-hydroxy-cinnamic acid	mixed	—	NC	0.0063

[a] Data taken from ref. 30.

[b] Pyruvate-dependent $^{14}CO_2$ fixation by mesophyll chloroplast preparations was as described in Materials and Methods; values are the average of at least three separate experiments.

[c] C, competitives; UC, uncompetitive; NC, non-competitive.

[d] Calculated from axis intercept according to type of inhibition.

included for comparison. In general, the two systems appear quite different; the apparent K_m (pyruvate) was roughly 10-fold higher with chloroplasts and the observed K_i values were orders of magnitude greater. In the case of pyruvate analogues, α-ketoisovalerate and α-ketoisocaproate were inhibitory with chloroplasts but are reported to have no effect on mitochondria.

CONCLUSIONS

This study is the first report directly demonstrating transport of pyruvate in mesophyll chloroplasts of a C_4 plant. As such, it is consistent with previous studies that suggest pyruvate transport to occur [2, 3] and strengthens proposals for carbon flow in C_4 plants which require pyruvate transport into the chloroplast. There is another theory for C_4 photosynthesis which does not involve intercellular compartmentation of the C_3 and C_4 pathways [8]. This theory does not necessitate the transport of pyruvate across the mesophyll chloroplast envelope, since pyruvate would be formed and consumed within the chloroplast stroma. The evidence reported here for a pyruvate carrier is supportive of the former theory.

Evidence for carrier-mediated transport

The following lines of evidence suggest the existence of a pyruvate carrier: (1) kinetics of uptake could be resolved at room temperature and appear to obey Michaelis-Menten kinetics (Fig. 4); (2) transport is specifically inhibited by a number of pyruvate analogues and α-cinnamic acid derivatives (Tables I and II); (3) initial uptake velocity is dependent on ionic strength (data not shown); (4) uptake is blocked

by the sulfhydryl reagent mersalyl (Figs. 13 and 14), and (5) transport is highly temperature sensitive (Fig. 15) and is not observed in spinach (C_3) chloroplasts (data not shown). The presence of a carrier is quite logical, since at pH 8.0, the ratio of conjugate base to acid is on the order of $3 \cdot 10^5$.

Nature of pyruvate transport

Kinetic constants extrapolated from experiments performed at low concentrations of [^{14}C]pyruvate suggest that the uptake observed in the dark (i.e. in the absence of energy) can account for the observed rates of pyruvate metabolism in the light. The apparent K_m (pyruvate) and V so derived are of the same order of magnitude as those obtained for metabolism of pyruvate in the light. The apparent K_m (pyruvate) for transport and metabolism are higher than the reported K_m (pyruvate) of pyruvate-orthophosphate dikinase (250 μM [32]). This would be expected since the internal/external concentration of pyruvate is always less than one; in order to maintain an internal pool of 250 μM pyruvate, an external concentration on the order of 600–800 μM would be required and hence the apparent K_m (pyruvate) for metabolism will be higher.

Net uptake of pyruvate appears to occur largely by permeation of the anion and may be dependent on the membrane potential existing across the envelope. This model is based on the observed swelling in potassium pyruvate plus valinomycin, the inhibitory effect of other permeant anions, predictable effects of conditions which are assumed to modify the membrane potential, and the ability of permeant anions to "chase" pyruvate out of the chloroplast. These conclusions are qualitatively substantiated by experiments with the membrane permeant cation tetraphenylmethylphosphonium.

Relation of uptake in dark to metabolism in the light

Data has been presented in Tables I and II that compounds which inhibit [^{14}C]pyruvate uptake in the dark also inhibit pyruvate metabolism in the light, indicating that the transport studied is related to that which is involved in metabolism. The results suggest that uptake of the pyruvate anion is via an electrogenic carrier; the coupling of pyruvate uptake to fluxes of other photosynthetic intermediates will be described in a subsequent paper.

ACKNOWLEDGEMENTS

This research was supported by the College of Agriculture and Life Sciences, University of Wisconsin, Madison and by the University of Wisconsin Research Committee, with funds from the Wisconsin Alumni Research Foundation, and by N.S.F. grant BMS-74-09611. S.C.H. is the recipient of an N.I.H. Traineeship in Genetics. The authors thank Drs. H. A. Lardy and S. J. Gately for helpful discussion, and Dr. J. Adler for reading the manuscript.

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