Metabolite Diffusion into Bundle Sheath Cells from C₄ Plants

RELATION TO C₄ PHOTOSYNTHESIS AND PLASMODESMATAL FUNCTION

Hendrik Weiner¹, James N. Burnell, Ian E. Woodrow, Hans W. Heldt¹, and Marshall D. Hatch*
Division of Plant Industry, CSIRO, GPO Box 1600, Canberra City A.C.T. 2601, Australia

Received for publication April 4, 1988

ABSTRACT

The present studies provide the first measurements of the resistance to diffusive flux of metabolites between mesophyll and bundle sheath cells of C₄ plants. Species examined were Panicum miliaceum, Urochloa panicoides, Atriplex spongiosa, and Zea mays. Diffusive flux of metabolites into isolated bundle sheath cells was monitored by following their metabolic transformation. Evidence was obtained that the observed rapid fluxes occurred via functional plasmodesmata. Diffusion constants were determined from the rate of transformation of limiting concentrations of metabolites via cytosolic enzymes with high potential velocities and favorable equilibrium constants. Values on a leaf chlorophyll basis ranged between 1 and 5 micromoles per minute per milligram of chlorophyll per millimolar gradient depending on the molecular weight of the metabolite and the source of bundle sheath cells. Diffusion of metabolites into these cells was unaffected by a wide variety of compounds including respiratory inhibitors, monovalent and divalent cations, and plant hormones, but it was interrupted by treatments inducing cell plasmolysis. The molecular weight exclusion limit for permeation of compounds into bundle sheath cells was in the range of 850 to 900. These cells provide an ideal system for the quantitative study of plasmodesmatal function.

A key feature of C₄ plants is the differential partitioning of photosynthetic reactions between mesophyll and bundle sheath cells (21). As a consequence, there is a need for rapid intercellular transport of certain metabolites between these cells. The prime function of C₄ photosynthesis is to concentrate CO₂ in bundle sheath cells (1, 17, 18). Therefore, the mesophyll-bundle sheath cell interface must be permeable enough to permit the appropriate flux of metabolites while, on the other hand, providing a sufficient diffusion barrier to prevent unacceptable losses of the inorganic carbon concentrated in bundle sheath cells. The requirement for rapid fluxes of metabolites between these cells has been causally linked with the observed high frequency of plasmodesmata connecting mesophyll and bundle sheath cells (22) and with the occurrence of larger pools of those metabolites that diffuse between these cells (18, 21). An estimate of the resistance to diffusion of metabolites between mesophyll and bundle sheath cells has been made based on assumptions about plasmodesmatal frequency (21). With this value for resistance, concentration gradients of the order of 10 mm between these cells would be necessary to sustain maximum rates of photosynthesis. Subsequent direct measurements of cellular pools of metabolites in Zea mays indicate potential gradients in the range of 5 to 10 mm (23, 27). This derived value for mesophyll-bundle sheath cell resistance has also been applied to different models and given estimated diffusive losses of stored inorganic carbon from bundle sheath cells of the order of 10% of the CO₂ originally generated by C₄ acid decarboxylation (14, 21).

Clearly, a more precise analysis of C₄ pathway function would require more reliable direct measurements for the diffusion characteristics of the mesophyll-bundle sheath cell interface. It is difficult to conceive how such measurements might be obtained with intact leaves but, as recently pointed out (3), isolated bundle sheath cells may provide an alternative system. Rapid flux of C₄ acids or C₃ compounds into isolated bundle sheath cell strands, as judged by resulting rates of metabolism or photosynthesis, have been observed in several separate studies (4, 6, 19, 26). Responses to the addition of various other metabolites and inhibitors have also been observed, including compounds as large as adenylates and NAD (3, 4, 9).

During the present study we examined the diffusion of various metabolites into isolated bundle sheath cell strands from C₄ species and a procedure was developed for measuring diffusion constants for this process. This diffusive flux apparently occurred via plasmodesmata. The results are considered in relation to the function of C₄ photosynthesis.

MATERIALS AND METHODS

Materials. Bundle sheath cells or protoplasts were prepared from leaves harvested from plants grown in soil in a naturally illuminated glasshouse maintained between 20 and 30°C. The species examined were Panicum miliaceum, Urochloa panicoides, Atriplex spongiosa, and Zea mays. Biochemicals and reagent enzymes were obtained from Boehringer-Mannheim, Australia, or Sigma Chemical Company.

Preparation of Bundle Sheath Cells and Protoplasts. Bundle sheath cell strands were prepared by a modification of the procedure of mechanical blending of leaf tissue (19) and purification by filtration (3). Top expanded leaves from 15 to 25 d old plants were harvested and the lamina free of midrib were floated on water for about 30 min to allow full turgidity to be regained. About 10 g of this tissue was sliced transversely into 1 to 2 mm sections and then homogenized in a Sorvall Omnimixer with 120 ml of 0.3 M sorbitol containing 20 mM Hepes-KOH (pH 7.5), 0.5 mM potassium phosphate, 4 mM isocitrate, 10 mM KCl, and 0.2% (w/v) bovine serum albumin. The tissue was blended for about 10 s at setting 6 (60% of line voltage) and then periods of about 20 s at setting 4 until microscopic examination showed that the majority of bundle sheath cells occurred in single strands detached from epidermis and free of mesophyll cells. Much of the epidermal material and remaining larger pieces of leaf tissue were then removed by sequential filtration of the homogenate through nylon nets of aperture 1 mm and then 0.6 mm. The remaining strands of bundle sheath cells were collected

¹ Present address: Institut für Biochemie der Pflanze, Universität Göttingen, 3400 Göttingen, FRG.
on Miracloth (Calbiochem, Sydney) and washed by resuspension in 100 mL of medium (referred to below as the wash buffer) containing 0.3 m sorbitol, 20 mM Hepes-KOH (pH 7.5), 0.5 mM potassium phosphate, and 10 mM KCl. The cells were allowed to settle in a 100 mL measuring cylinder for a period of about 10 min at 0 to 5°C and the supernatant containing contaminating epidermal tissue (which settles more slowly) was removed. This step was repeated twice. This gentle washing step was critical for removing traces of enzymes released into the medium from broken cells. For the present studies, trace contamination by enzymes released from broken cells (as little as 1% of the total enzyme present in cells) generate critical interference with the assay procedure. The washed cells were gently resuspended in the wash medium (diluted to give a Chl concentration of between 20 and 30 μg mL⁻¹) and stored at 0°C prior to sampling for the experiments described below.

Protoplasts from the bundle sheath cells of P. miliiaceum were prepared essentially as previously described (10) using the tissue digestion procedure involving incubation for 18 h at 18 to 19°C and the ‘Purification II’ procedure. All media contained 0.1% (w/v) bovine serum albumin and the purified protoplasts were suspended and assayed in a medium containing 0.5 mM sucrose, 5 mM Hepes-KOH (pH 7.5), and 0.1% (w/v) bovine serum albumin. This medium was close to isopycnic with respect to bundle sheath cell protoplasts. For all steps where protoplasts were centrifuged to the bottom of the tubes they were collected on a high-density cushion of medium containing 0.5 mL of medium (referred to below as the wash buffer) and 0.5 to 1% (w/v) dextran-40.

Measurement of Diffusion Flux by Metabolite Transformation in Cells was studied with isolated protoplasts or protoplasts. Samples of bundle sheath cell strads (usually 800 μL in 0.3 m sorbitol, 20 mM Hepes-KOH [pH 7.5], 0.5 mM K-phosphate, 10 mM KCl) or protoplasts (in 0.5 mM sucrose, 5 mM Hepes-KOH [pH 7.5], 0.1% [w/v] bovine serum albumin and also 1 mM CaCl₂ except for O₂ exchange studies) containing between 20 and 30 μg Chl mL⁻¹ were warmed to 25°C. For most studies cells were incubated for 10 min with pronase at this stage to inactivate traces of enzyme in the suspending medium (see below). Reactions were then started by adding 200 μL of substrate mixtures and shaken at 30 s intervals before stopping after periods of 1.3 to 3 min by the addition of 0.2 mL of 30% (w/v) TCA. Stirling of cells is critical, particularly with lower concentrations of substrates. Where the cells were not treated with pronase (pronase) activity due to enzyme released into the suspending medium was assessed by running identical reactions with medium from which cells or protoplasts had been removed by centrifugation. Alternatively, this activity was eliminated by preincubating cells with pronase (see above). For malate dehydrogenase-based reactions, pretreatment for 10 min at 25°C with 0.1% (w/v) pronase was sufficient but 0.5 to 1% (w/v) pronase (pretreated on a Sephadex G-25 column to remove small mol wt contaminants) was required for alanine aminotransferase.

This concentration was only partially effective with triose phosphate isomerase. After standing the acidified reaction mixtures for at least 60 min at 0°C with intermittent shaking, solid material was removed by centrifugation at 10,000g for 2 min. A 0.9 mL sample of the clear supernatant was neutralized to about pH 7.5 (or pH 8.7 for malate dehydrogenase-based assays, see below) by the addition of 0.1 mL of 1 M Hepes buffer and then a predetermined amount of 2 M KOH. Samples of these solutions were then assayed for product formation by the procedures described below.

NADH-mediated reduction of OAA² to malate via malate dehydrogenase was measured by the production of NADH at 340 nm. The procedure is 0.1% (w/v) dextran-40. NADH used as a substrate for these studies was initially heated at 70°C for 25 min in 50 mM KHCO₃ (pH 10.0) to destroy traces of contaminating NAD (24). After incubating reactions as described above they were stopped with TCA (0°C, 60 min) also destroyed NADH remaining in the reaction mixtures (24). Reactions were then neutralized by the addition of 0.2 mL of 0.66 M Tricine-KOH (pH 8.7) followed by about 0.15 mL of 2 M KOH to give a final pH of about 8.5. The NAD in this extract was determined by adding samples (up to 0.4 mL) to 0.6 mL of a mixture of 0.2 μL pyrophosphate-NAOH buffer (pH 8.8) containing 1% (w/v) semicarbazide-HCl and 0.1 M ethanol in a total volume of 1 mL. Measurement was initiated by adding 10 μL of yeast alcohol dehydrogenase (5–10 units) and the increase in absorbance at 340 nm was recorded.

The basis for determining other metabolite transformations was as follows: (a) conversion of alanine plus 2-oxogluturate to pyruvate plus glutamate via alanine aminotransferase was measured by pyruvate formation with lactate dehydrogenase (19), (b) conversion of aspartate plus 2-oxoglutarate to OAA plus glutamate via aspartate aminotransferase was measured by OAA formation with malate dehydrogenase, and (c) conversion of GAP to DHAP via triose-P isomerase was measured by using glycerol 3-P dehydrogenase in an assay system containing 50 mM Hepes-KOH (pH 7.5) and 0.2 mM NADH. The change in absorbance at 340 nm was determined after adding glyceraldehyde 3-P dehydrogenase (2 units).

Determination of Diffusion Constants. Diffusion constant values were estimated from the observed velocities of the reactions described above at limiting substrate concentrations. With cytosolic enzymes with limited rates (less than 1 Vₘₐₓ), it was assumed that reaction rates are very largely limited by inward diffusion when the external substrate concentration is highly limiting. The basis of procedures for plotting these data to provide estimates of the diffusion constant, and the theory behind these treatments, are discussed in “Results” and “Appendix.”

Molecular Weight Exclusion Limit for Diffusion into Bundle Sheath Cells. The procedure used is described in detail elsewhere (2). It is based on measuring the effect of dyes-inhibitors of varying mol wt on the capacity of intact cells to transform alanine to pyruvate via alanine aminotransferase. Procion Yellow H-5G (Reactive Yellow 2, provided by ICI Australia Pty Ltd, Melbourne, Australia) was reacted with various amines and polypeptides to give a series of products. Cells were preincubated with a series of these derivatives for 5 min at 25°C and then assayed for their capacity to convert alanine to pyruvate. Lack of inhibition was taken as an indication that the inhibitor had not penetrated the cells.

RESULTS AND DISCUSSION

Permeability of Bundle Sheath Cells and Protoplasts to Metabolites. Flux of metabolites into bundle sheath cells and protoplasts was monitored by measuring the metabolic transformation of the compounds via cytosolic enzymes. For this purpose, metabolites were chosen which are substrates for enzymes known to occur in reasonably high levels in the cytosol. Table I provides information about the metabolites and associated enzyme systems used. Measured in the forward direction all these enzymes offer a high potential velocity (Vₘₐₓ) in the cells. For comparison, these activities are at least an order of magnitude higher than the maximum photosynthesis rates of C₄ leaves (approximately 5 μmol min⁻¹ mg⁻¹ Chl) and much higher than the activities of many other common cytosolic enzymes. However, the equilibrium constants for these reactions vary widely as also does the proportion of the total cell activity actually present in the cytosol. As will be discussed later, the cytosolic activity of triose-P isomerase in some cells may be too low for use in this procedure.

Alanine and 2-oxoglutarate rapidly diffused into isolated Pan-
**Table 1. Metabolites and Enzyme Systems Used for Studies of Diffusion Flux into Bundle Sheath Cells and Protoplasts**

<table>
<thead>
<tr>
<th>Enzyme and Reaction</th>
<th>Characteristic of Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>$3 \times 10^4$</td>
</tr>
<tr>
<td>OAA + NADH ⇌ Malate + NAD</td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>0.67</td>
</tr>
<tr>
<td>Ala + 2OG ⇌ Pyruvate + Glu</td>
<td></td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>0.15</td>
</tr>
<tr>
<td>Asp + 2 - OG ⇌ OAA + Glu</td>
<td></td>
</tr>
<tr>
<td>Triose-P isomerase</td>
<td>24</td>
</tr>
<tr>
<td>GAP ⇌ DHAP</td>
<td></td>
</tr>
</tbody>
</table>

$^a$2-OG, 2-oxoglutarate.  $^b$Activities in extracts of bundle sheath cells from *P. miliaceum* and *U. panicoides* (in parentheses). Data from Burnell and Hatch (3) and our unpublished results.  $^c$Based on analysis of intracellular location made in earlier studies (7, 20) and analysis of the subcellular distribution of enzymes in *P. miliaceum* protoplasts in the present study (see text).

*

**icun miliaceum** bundle sheath cells as judged by the rate at which these substrates were transformed to pyruvate (Fig. 1). By contrast, protoplasts from these cells were apparently essentially impermeable to these substrates. With cells, the maximum rate of pyruvate production from alanine plus 2-oxoglutarate was about 15 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \text{ Chl} \) and the \( K_m \) for 2-oxoglutarate was about 4 \( \mu \text{M} \). After correcting for activity due to enzyme released in the medium ('supernatant' in Fig. 1) pyruvate production attributable to intact protoplasts was negligible.

The presence in the suspending medium of even a small proportion of total enzyme results in high blanks relative to the activity due to cells (see "Materials and Methods"). Furthermore, this so-called supernatant activity reflects the true \( K_m \) for substrates so it shows maximum activity at much lower substrate concentrations than seen for the activity due to intact cells (Fig. 1). For cells, repeated washing by gravity sedimentation reduced this supernatant blank and in later experiments it was eliminated by treating cells with the proteolytic enzyme pronase. With protoplasts it was more difficult to reduce this supernatant blank. Some critical factors in this respect were recovery by sedimenting onto a cushion of highly density medium, gentle manipulation and resuspension, and running reactions in near isopycnic medium (see "Materials and Methods").

Further evidence that bundle sheath cells are highly permeable to metabolites compared with protoplasts was provided by comparing rates of PGA-dependent \( \text{O}_2 \) evolution. Bundle sheath cell strands from *P. miliaceum* evolved \( \text{O}_2 \) at rates of between 1 and 2 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \text{ Chl} \) in different preparations. By contrast, no \( \text{O}_2 \) was evolved from illuminated protoplasts provided with PGA. However, breaking protoplasts (passing through an 80 \( \mu \text{m} \) net) resulted in the immediate appearance of PGA-dependent \( \text{O}_2 \) evolution by the released chloroplasts. Similar results have been reported for mesophyll protoplasts from *Zea mays* (8).

As discussed in the introduction, the occurrence of a large number of plasmodesmata spanning the mesophyll-bundle sheath cell wall of \( \text{C}_4 \) plants has been linked with the requirement for rapid flux of metabolites between these cells. It seems most likely that plasmodesmata must account for the relatively high permeability of isolated bundle sheath cell strands to various metabolites. Presumably, during the preparation of protoplasts these structures would be destroyed due to fusing of the plasmamembrane at the points where plasmodesmata were originally attached. Further evidence that metabolites diffuse via plasmodesmata is provided below.

**Diffusion Constants for Metabolite Flux.** For the present purposes, the term diffusion constant \( (k_d) \) is used to describe the rate of diffusion of a particular metabolite into cells from a particular species with a concentration gradient of 1 \( \text{mm} \). Using the metabolites and enzyme systems described in Table I, our approach was to increase the limitation due to metabolite diffusion into bundle sheath cells by decreasing the substrate concentration. Figure 2 shows the responses to decreasing OAA and NADH on the rate of NAD formation via malate dehydrogenase in bundle sheath cells from *P. miliaceum*. In these experiments the activity due to malate dehydrogenase released into the medium was eliminated by preincubating the cells with pronase (see "Materials and Methods"). A minimum estimate of the diffusion constant (expressed as a rate for a gradient of 1 \( \text{mm} \), assuming the internal concentration is near zero) can be obtained from the initial slope of the substrate response curve. For the experiment shown in Figure 2 the values obtained for OAA and NADH were about 9 and 4.5 \( \mu \text{mol min}^{-1} \text{ mg}^{-1} \text{ Chl} \text{ mm}^{-1} \), respectively.

Plots of the reciprocal of reaction velocity against the reciprocal of OAA or NADH concentration gave an approximately linear response with apparent \( K_m \) values of 0.36 and 0.55 \( \text{mm} \), respectively (Fig. 2). These values are more than an order of magnitude

![Graph](image-url)
higher than the $K_m$ values obtained for cytosolic malate dehydrogenase isolated from *P. miliaceum* bundle sheath protoplasts (20 and 25 $\mu$M for OAA and NADH, respectively). We take this as an indication that the metabolism of these compounds was very substantially limited by their flux into bundle sheath cells.

The diffusion constant for metabolite flux into bundle sheath cells is more precisely determined by plotting the ratio of substrate concentration ($s$) to velocity ($v$) against velocity. At lower substrate concentrations this plot is linear and an approximation of the diffusion constant is given by the reciprocal of the intercept of this line on the axis (Fig. 2). It is accepted that velocity cannot be totally limited by diffusion even at very low external substrate concentrations. The values obtained for oxaloacetate and NADH were 10.4 and 6.3 $\mu$mol min$^{-1}$ mg$^{-1}$ Chl mm$^{-1}$, respectively. The theoretical basis for this treatment is given in the “Appendix” to this paper together with some consideration of the effect of $K_m$ and substrate $K_m$ on the estimation of diffusion constant.

Table II summarizes data for diffusion constant determinations for various metabolites. For both OAA and NADH higher diffusion constant values were obtained for flux into *P. miliaceum* bundle sheath cells compared with the cells from other species (Table II). Notably, the diffusion constants for NADH with cells from particular species was always less than that obtained for OAA with an average value of about 45% less. This difference can be very largely explained by the lower diffusion coefficient of NADH due to its higher mol wt. Diffusion coefficients are inversely proportional to the cube root of the ratio of mol wt so that the coefficient for NADH should be about 40% lower than the coefficient for OAA.

As noted above, the most accurate estimates of diffusion constants will be provided by coupling of metabolite flux to cytosolic reactions with a high potential velocity and an equilibrium favoring the direction of assay. The adverse effect of an unfavorable equilibrium or lower $V_{max}$ values is demonstrated in the treatments developed in the “Appendix.” By these criteria, malate dehydrogenase provides an ideal system. The other favorable factor is a low $K_m$ for the diffusing metabolite and this is also demonstrated in the treatment developed in the “Appendix.” For each enzyme the intercept on the $s/v$ axis gives a good estimate of $1/K_m$ (reciprocal of diffusion constant) provided the $K_m$ for the particular substrate is low (or more specifically $K_m/V_m$ is small). This $K_m$ factor could explain why diffusion constants for 2-oxoglutrate (determined with alanine aminotransferase coupling) were similar to those obtained for OAA but the values for alanine were much lower (Table II). With alanine aminotransferase isolated from bundle sheath cells of the species used in Table II, the $K_m$ for 2-oxoglutate ranged between 30 and 80 $\mu$M compared with a range between 3 and 6.5 mm for the $K_m$ of alanine (determined during the present study with a saturating concentration of the nonvariable substrate). Notably, the apparent $K_m$ for 2-oxoglutarate with intact cells was much higher (about 2 mm) whereas the apparent $K_m$ for alanine was similar to that for the isolated enzyme.

Estimates of the diffusion constants for 2-oxoglutrate and oxaloacetate were also made by coupling to the cytosolic aspartate aminotransferase of the PEP carboxyl kinase-type C$_4$ species *U. panicoides*. For both metabolites the values obtained for the diffusion constant were similar to those shown in Table II using malate dehydrogenase and alanine aminotransferase for oxaloacetate and 2-oxoglutarate, respectively.

The considerations in the “Appendix” also suggest that the diffusion constant for GAP could be determined by coupling to triose-P isomerase (low $K_m$ for GAP of about 0.3 mm and an equilibrium favoring DHAP formation). In practice, we encountered considerable technical problems with this system largely related to the very high activity of released enzyme. This activity was only partially and variably inactivated by pretreatment of cells with pronase. Furthermore, this released enzyme activity was much higher relative to the activity due to cells compared with other enzymes we used. This was probably due to the very high total activity of triose-P isomerase in these cells (see Table I) combined with the relatively low proportion of this activity that was located in the cytosol.

However, for three of the species examined a reproducible estimate of the diffusion constant for GAP was obtained. These values were similar to those obtained for OAA and 2-oxoglutarate (Table II). Reliable values were not obtained with *P. miliaceum* cells. Significantly, when the subcellular distribution of triose-P isomerase was determined by fractionating extracts of *P. miliaceum* bundle sheath cell protoplasts, essentially all the enzyme was found associated with chloroplasts (determined by comparison with NADP glyceraldehyde 3-P dehydrogenase as a marker enzyme for chloroplasts).

In summary, the results presented in Table II indicate that compounds of similar mol wt give similar diffusion constants with cells from the same species. There is a link with mol wt roughly in accordance with the theoretical effect on the diffusion coefficient, but no evidence for any selectivity in the diffusion process. The diffusion constants for particular compounds vary between species, possibly due to the varying occurrence of plasmodesmata in the cell wall. Average values for the data in Table...
INTERCELLULAR DIFFUSION OF METABOLITES IN C₄ PHOTOSYNTHESIS

Table II. Diffusion Constants for Flux of Metabolites into Bundle Sheath Cells from Leaves of Different C₄ Species

Results are given for diffusion constants calculated from the reciprocal of the intercept of the plot of s/v against v (see text). Where replicates were obtained the mean, the standard deviation, and the number of determinations (in parentheses) are given. For further details of the procedures for determining diffusion constants see the text, "Materials and Methods," and "Appendix I."

<table>
<thead>
<tr>
<th>Species</th>
<th>OAA</th>
<th>NADH</th>
<th>2-Oxoglutarate</th>
<th>Alanine</th>
<th>GAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol min⁻¹ mg⁻¹ Chl mm⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. miliaceum</td>
<td>10.1 ± 1.3 (3)</td>
<td>6.5 ± 1.9 (3)</td>
<td>8.0 ± 0.4 (3)</td>
<td>3.4 ± 0.2 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.4⁷</td>
<td>6.3⁷</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U. panicoides</td>
<td>7.1</td>
<td>3.5</td>
<td>4.5 ± 0.3 (2)</td>
<td>1.6 ± 0.4 (2)</td>
<td>5.5</td>
</tr>
<tr>
<td>Z. mays</td>
<td>6.3 ± 1.3 (2)</td>
<td>2.7 ± 0.8 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. spongiosa</td>
<td>7.8 ± 1.2 (2)</td>
<td>4.0 ± 0.3 (2)</td>
<td>4.5</td>
<td>0.9</td>
<td>4.3 ± 0.7 (2)</td>
</tr>
</tbody>
</table>

*In this experiment data were obtained by incubating reactions for 3 min with regular mixing (30 s intervals). The first set of data, shown as the average of three determinations, were obtained with 1.33 min incubation times but no stirring during the incubation period.

Table III. Summary of Diffusion Constant Data

<table>
<thead>
<tr>
<th>Species</th>
<th>Compounds of mol wt 131-170⁴</th>
<th>NADH (mol wt 665)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bundle sheath Chl</td>
<td>Leaf Chl</td>
</tr>
<tr>
<td></td>
<td>μmol min⁻¹ mg⁻¹ Chl mm⁻¹</td>
<td></td>
</tr>
<tr>
<td>P. miliaceum</td>
<td>9 ± 1.1</td>
<td>5.4</td>
</tr>
<tr>
<td>U. panicoides</td>
<td>5.7 ± 1.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Z. mays</td>
<td>5.1 ± 1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>A. spongiosa</td>
<td>5.6 ± 1.1</td>
<td>3.3</td>
</tr>
</tbody>
</table>

*Mean of data for OAA, 2-oxoglutarate and GAP from Table II. ⁴Calculated from the distribution of leaf Chl between mesophyll and bundle sheath cells reported by Mayne et al. (25) and our unpublished data for A. spongiosa.

II are provided in Table III together with values for diffusion constants expressed on a total leaf Chl basis. For compounds in the mol wt range of 130 to 170 (OAA, 2-oxoglutarate, and GAP) mean values for diffusion constants expressed on the basis of bundle sheath cell Chl ranged between 5 and 9 μmol min⁻¹ mg⁻¹ Chl mm⁻¹. For NADH (mol wt 665) the range was 2.7 to 6.5. Diffusion constants based on whole leaf Chl ranged between 1.9 and 5.4 μmol min⁻¹ mg⁻¹ Chl mm⁻¹ for the lower mol wt compounds and 1.0 to 3.9 μmol min⁻¹ mg⁻¹ Chl mm⁻¹ for NADH.

Molecular Weight Exclusion Limit for Bundle Sheath Cell Permeability. Dye enzyme inhibitors of varying mol wt were used to assess the mol wt limit for permeability into bundle sheath cells. The method is described in detail elsewhere (2). Preliminary experiments using different dyes of varying mol wt gave an apparent exclusion limit of about 850 when monitored by OAA production via aspartate aminotransferase or pyruvate production via alanine aminotransferase in bundle sheath cells from Urochloa panicoides (results not shown). This result was confirmed by measuring inhibition of alanine aminotransferase-dependent pyruvate production in cells preincubated with polypeptide derivatives of the dye Reactive Yellow-2 of varying mol wt (Fig. 3). All derivatives (mol wt range 810-1100) completely inhibited alanine aminotransferase isolated from P. miliaceum bundle sheath cells. When strands of bundle sheath cells were preincubated with these various derivatives prior to assaying for their capacity to convert alanine to pyruvate inhibition was seen with compounds of mol wt of about 880 or less. Similar results were obtained with cells from four different C₄ species. The transition range was between mol wt of 860 and 920.

Also relevant is the observation that Evans Blue (mol wt 960) did not penetrate bundle sheath cells of P. miliaceum thus confirming the observation made earlier with U. panicoides bundle sheath cells (3). Notably, no plasmolysis was seen in P. miliaceum cells incubated with up to 1 M sucrose or sorbitol. However, plasmolysis was seen with 0.5 M PEG of mol wt about 1000 and 1500 suggesting that these compounds did not enter the cell. With PEG of mol wt 3500 cell wall collapse (cytorrhysis, see Ref. 5) was observed.

The mol wt exclusion limit we observed in the region of 800 to 900 is in a similar range to the limits determined by microscopic assessment of the movement of fluorescent compounds between cells of various tissues (13, 15, 28) but higher than the apparent limit in some other tissues (13). Notable in this connection is the conclusion of Terry and Robards (28) that the exclusion limit of compounds through plasmodesmata is almost certainly related primarily to the effective Stokes radius of the molecules rather than the mol wt as such. The similarity between the mol wt exclusion limit observed in the above studies and the values we obtained provides further support for the view that diffusion of metabolites into bundle sheath cells is via functional plasmodesmata.
Factors Affecting Metabolite Flux into Bundle Sheath Cells. The aim of this study was to identify possible effectors of plasmodesmatal function. Metabolite diffusion was monitored either by following pyruvate production in the presence of limiting 2-oxoglutarate or alanine, or OAA-dependent conversion of NADH to NAD with limiting NADH (see "Materials and Methods"). There was no significant inhibition of metabolite flux after preincubating the cells for 4 min with the following: 5% (v/v) ethanol, 100 mM KCl, EDTA, CaCl₂, tryptonate and dithiothreitol at up to 5 mM; MgCl₂, phenylalanine and histidine at up to 10 mM; indolacetic acid, gibberellic acid and FCCP at up to 50 μM, 19 μM kinetin, and 25 μM antimycin A. Substantial inhibition by higher concentrations of p-chloromercuribenzoate and CdCl₂ (which bind thiols and dithiols) was entirely attributable to inhibition of alane aminotransferase (the isolated enzyme was inhibited to a greater extent than the activity observed with intact cells).

The effect of varying pH on measured flux was similar to the effect of pH on the activity of isolated alane aminotransferase. Preincubation with proteinase K apparently partially inhibited flux into cells but several other proteinases had no effect. Pronase inhibited activity with bundle sheath cells from *Atriplex spongiosa* but not other species. Some of the above treatments were included because of earlier reports of inhibitory effects on plant preincubation with inhibitors of compounds. For instance Erwee and Goodwin (11, 12) reported that Ca²⁺ and Mg²⁺, and also the aromatic amino acids phenylalanine, tyrosine, and tryptonate, partially inhibited the intercellular transfer of fluorescent dyes. We did not observe inhibitory effects with any of these compounds.

Preincubating *P. miliaceum* bundle sheath cells with 0.18 and 0.35 m PEG-1500 (mol wt about 1500), which is known to cause plasmolysis of these cells, largely or completely inhibited the OAA-dependent conversion of NADH to NAD (results not shown). In the only trial done on the reversibility of this effect, flux was not only recovered after deplasmolysis but was actually increased compared with the original value. These results are consistent with metabolite flux being mediated via plasmodesmata which presumably would be modified or disrupted by plasmolysis. A similar inhibitory effect on the microscopically determined movement of fluorescent dyes after plasmolysis was noted earlier (12).

CONCLUDING COMMENTS

As outlined in the introduction, the diffusion characteristics of the interface between mesophyll and bundle sheath cells is a critical factor in the operation of photosynthesis in C₄ plants. During the present study the diffusion constants for flux of various metabolites into isolated bundle sheath cells were directly measured in the expectation that this would reflect the diffusion resistance of the mesophyll-bundle sheath cell interface in vivo. The values obtained ranged between 2 and 5 μmol min⁻¹ mg⁻¹ Chl mm⁻¹ for small mol wt compounds (mol wt 131–170) and 1–3.9 for NADH, expressed on a leaf Chl basis. These values are higher than the diffusion constant previously deduced by an indirect procedure for metabolite movement between mesophyll and bundle sheath cells (21). Taking an average value of 3 μmol min⁻¹ mg⁻¹ Chl mm⁻¹, the gradient required to give intercellular flux matching maximum photosynthesis would be about 2 mm⁻¹ using the model of Furbank and Hatch (14) the corresponding rate of leakage of inorganic carbon from bundle sheath cells would represent about 30% of released CO₂. Values for these parameters using the diffusion resistance deduced in the earlier study (21) were about 10 mm for the gradient and leak rate of stored CO₂ of about 10% (see introduction). Of course, it is possible that plasmodesmatal function may be modified during the isolation of bundle sheath cells. However, the reproducibility of the results with different preparations of cells from the same species, the relatively narrow range of values obtained with different species, and the similarity of our determination of mol wt exclusion limit with those obtained in earlier studies, would argue against this.

At least the mass movement of metabolites between cells of plants is assumed to proceed largely via these tubule-like structures located in the cell wall termed plasmodesmata (16). The present studies provide presumptive evidence that the flux of metabolites into isolated bundle sheath cells occurs via functional plasmodesmata. Previous studies of plasmodesmata function and the factors affecting this process have been limited by the necessity for using a tedious microscope-based method which provides no scope for quantitating flux measurements. We suggest that isolated bundle sheath cells from C₄ plants, and possibly isolated cells generally, provide a much more flexible system for the detailed studies of plasmodesmata-mediated flux. Where such measurements are linked to enzymic transformation of the diffusing metabolite the most critical requirements for an effective coupling enzyme are defined in the present study.

APPENDIX

In the accompanying paper the diffusion of various metabolites into bundle sheath cells is followed by measuring metabolism linked to three different enzymes. This appendix develops the theoretical basis for using the plot of the ratio of substrate concentration to velocity versus velocity to determine diffusion constants for the flux of these metabolites. Diffusion constants will be designated as *k₁, k₂, k₃*, etc. to distinguish the values for different substrates. These are equivalent to the term *k₅* used in the paper.

Malate Dehydrogenase. Consider the reaction catalyzed by malate dehydrogenase

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{Malate} + \text{NAD}^+
\]

in which the equilibrium strongly favors malate formation.

At a constant pH and NADH concentration the reaction velocity as oxaloacetate (OAA) is varied is given by:

\[
v = \frac{V_m \text{[OAA]}}{[\text{OAA}] + K_m}
\]

where *Vₘ*, *Kₘ*, and *Kₐp* are the apparent maximum velocity and Michaelis constant, respectively. When the reaction takes place within bundle sheath cell strands, the diffusion of the substrates from the suspending medium to the cytosol of the cells is described by:

\[
\nu_{dl} = k₁([\text{OAA}]_o - [\text{OAA}]_i);
\]

and

\[
\nu_{d₂} = k₂([\text{NADH}]_o - [\text{NADH}]_i);
\]

where *k₁* and *k₂* are constants describing the diffusion processes, and the subscripts *o* and *i* indicate substrate pools in the external medium and within the cells, respectively.

In the steady state, 

\[
v = \frac{V_m \text{[OAA]}}{[\text{OAA}] + K_m} = v + K_m + ([\text{OAA}] - v/k_1)
\]

where *Vₘ*, *Kₘ*, and *Kₐp* are approximately constant when [NADH]₀ ∼ [NADH]₁ and [malate] ∼ *Kₐp* (malate). Eq. 1 can be written as

\[
\text{[OAA]}_o = \frac{1}{v} \left(1 + \frac{V_m \text{[OAA]}}{[\text{OAA}] + K_m} - v\right)
\]

When *v* ∼ *Vₘ*, Eq. 2 approaches
[OAA]_p\( \frac{v}{\nu} \approx \frac{1}{k_1} + \frac{K_m}{V_m} - \frac{\nu}{k_1 V_m}\)  

Under these conditions, a plot of \([OAA]_p\( \frac{v}{\nu} \) versus \(v\) intercepts the abscissa at \(1/k_1 + K_m/V_m\). Since, in the present experiments, \(K_m/V_m\) is negligible, the intersection should be approximately \(1/k_1\). The equation with a similar form to Eq. 3 can be written for \([NADH]_p\), if it is assumed that \([OAA]_p\( \frac{v}{\nu} \) \(> K_m\)(OAA)

\[\frac{[NADH]_p}{\nu} \approx \frac{1}{k_2} + \frac{K_m}{V_m} - \frac{\nu}{k_2 V_m}\]

A plot of \([NADH]_p\( \frac{v}{\nu} \) versus \(v\) intercepts the abscissa at approximately \(1/k_2\), because, under the conditions used \(K_m/V_m\) is negligible, \(1/k_2 + K_m/V_m\).

**Triosephosphate Isomerase.** Consider the reaction catalyzed by triosephosphate isomerase.

glyceraldehyde 3-P (GAP) \(\Rightarrow\) dihydroxyacetone 3-P (DHAP)

in which the equilibrium constant for this direction is 24. Under conditions where \([DHAP] < K_m\) (DHAP) and \([GAP] < K_m\) (GAP) the reaction velocity is approximated by:

\[v_r \approx k_r[\text{GAP}] - k_r[\text{DHAP}]\]

where \(k_r\) and \(k_i\) are rate constants describing the forward and reverse reactions, respectively. When this reaction takes place within the cytoplasm of the bundle sheath cells, the diffusion of DHAP and GAP between this compartment and the external solution is described by:

\[v_{dl} = k_i[\text{GAP}] - v_{dl} = k_i[\text{DHAP}] - [\text{DHAP}]\]

where \(k_i\) and \(k_i\) are constants describing the diffusion processes. In the steady state, \(v_i = v_{dl} = v\), and

\[\frac{[\text{GAP}]}{v} - \frac{[\text{DHAP}]}{v} \approx \frac{1}{k_f} + \frac{1}{k_i} + \frac{1}{k_2 K}\]

where \(k = k_f k_i\), \((K\) is the equilibrium constant for the triosephosphate isomerase reaction). When \([DHAP]\)/\(K \ll [GAP]\), and as \(1/k_f < 1/k_i + 1/k_2 K\), the value for \((1/k_i + 1/k_2 K)\) is approximated by the intercept on the abscissa of the plot of \([GAP]/v\) versus \(v\). This point is made against \(v\) because the above equations are valid only as \(v \rightarrow 0\) (low substrate) and extrapolation will give a value of \(1/k_i + 1/k_2 K\) under these conditions.

For the triosephosphate isomerase reaction \((K = 24)\) and assuming \(k_i \approx k_i\), \(1/k_i < 1/k_i\), and hence the intercept \(1/k_i\).

**Alanine Aminotransferase.** Consider the reaction catalyzed by alanine aminotransferase.

\(\text{Ala} + \text{2-oxoglutarate} (2-\text{OG}) \Rightarrow \text{Glu} + \text{Pyruvate} (\text{Pyr})\)

When \([2-\text{OG}] \gg K_m\) (2-OG), and \([\text{Ala}], [\text{Glu}], \) and \([\text{Pyr}]\) are less than their respective \(K_m\) values

\[v_r = k_r[\text{Ala}] - k_i[\text{Glu}][\text{Pyr}]\]

where \(v_r\) is the reaction velocity, \(k_r\) is the apparent first order rate constant for varying alanine and \(k_i\) is the apparent second order rate constant for the reverse reaction. The diffusion of the substrates and products of this reaction between the external medium and the cytoplasm of the bundle sheath cells is described by the following equations:

\[v_1 = k_1[\text{Ala}] - [\text{Ala}]\);

\[v_2 = k_2[\text{2-OG}] - [\text{2-OG}]\);

\[v_3 = k_3[\text{Glu}] - [\text{Glu}]\);

\[v_4 = k_4[\text{Pyr}] - [\text{Pyr}]\];

where \(k_1, k_2, k_3,\) and \(k_4\) are constants describing the diffusion of the respective substrates and products and the subscripts \(o\) and \(i\) indicate substrate pools in the external medium and within the cells, respectively.

In the steady state, \(v_1 = v_2 = v_3 = v_4 = v\), and if only small amounts of product are allowed to accumulate (i.e. \([\text{Pyr}]\) and \([\text{Glu}] \approx 0\) then

\[\frac{[\text{Ala}]}{v} \approx \frac{v_k}{k_f^{\text{Ala}} k_i k_k} + \frac{1}{k_f^{\text{Ala}}} + \frac{1}{k_i}\]

When \([\text{Ala}] \gg K_m\) (Ala), a similar equation can be written for \([2-\text{OG}]\).

\[\frac{[2-\text{OG}]}{v} \approx \frac{v_k}{k_f^{\text{2-OG}} k_i k_k} + \frac{1}{k_f^{\text{2-OG}}} + \frac{1}{k_i}\]

where \(k_f^{\text{2-OG}}\) is the apparent first order rate constant when 2-oxoglutarate is varied. Plots of \([\text{Ala}]\) versus \([2-\text{OG}]/v\) versus \(v\), therefore, intercept the abscissa at \((1/k_f^{\text{Ala}} + 1/k_i + 1/k_f^{\text{2-OG}} + 1/k_i\), respectively. Since \(K_m^{\text{Ala}} = V_m^{\text{Ala}} k_f^{\text{Ala}}\) and \(K_m^{\text{2-OG}} = V_m^{\text{2-OG}} K_m^{\text{2-OG}}\) these intercepts on the abscissa become \(1/k_i + K_m^{\text{Ala}} V_m^{\text{Ala}} + 1/k_i + K_m^{\text{2-OG}} V_m^{\text{2-OG}}\), respectively.

In the present studies the intercept for varying 2-oxoglutarate would approximate to \(1/k_f\) (the reciprocal of the diffusion constant) since the \(K_m^{\text{2-OG}}\) is relatively low (about 50 \(\mu\)M, see test) and

\[\frac{K_m^{\text{OG}}}{V_m} \ll \frac{1}{k_f^{\text{OG}}} + \frac{1}{k_i}\]

However, the \(K_m^{\text{Ala}}\) is high (about 5–10 mM) so that the intercept of the above plot will substantially underestimate the diffusion constant even at relatively high \(V_m\). A treatment very similar to that developed above would apply for metabolite flux coupled to the enzyme aminotransferase.

**LITERATURE CITED**


