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A New Mechanism for the Regulation of Stomatal Aperture Size in Intact Leaves[1]

Accumulation of Mesophyll-Derived Sucrose in the Guard-Cell Wall of *Vicia faba*

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At various times after pulse-labeling broad bean (*Vicia faba* L.) leaflets with $^{14}CO_2$, whole-leaf pieces and rinsed epidermal peels were harvested and subsequently processed for histochemical analysis. Cells dissected from whole leaf retained apoplastic contents, whereas those from rinsed peels contained only symplastic contents. Sucrose (Suc)-specific radioactivity peaked (111 GBq mol^{-1}) in palisade cells at 20 min. In contrast, the ^{14}C content and Suc-specific radioactivity were very low in guard cells for 20 min, implying little CO_2 incorporation; both then peaked at 40 min. The guard-cell apoplast had a high maximum Suc-specific radioactivity (204 GBq mol^{-1}) and a high Suc influx rate (0.05 pmol stoma^{-1} min^{-1}). These and other comparisons implied the presence of (a) multiple Suc pools in mesophyll cells, (b) a localized mesophyll-apoplast region that exchanges with phloem and stomata, and (c) mesophyll-derived Suc in guard-cell walls sufficient to diminish stomatal opening by approximately 3 μm. Factors expected to enhance Suc accumulation in guard-cell walls are (a) high transpiration rate, which closes stomata, and (b) high apoplastic Suc concentration, which is elevated when mesophyll Suc efflux exceeds translocation. Therefore, multiple physiological factors are integrated in the attenuation of stomatal aperture size by this previously unrecognized mechanism.

For many years sugars played a prominent role in the explanations of stomatal movements. The classical theory (starch → sugar during stomatal opening, and vice versa) invoked an osmotic role for sugars within guard cells sufficient to create the requisite turgor for stomatal opening. This theory was based on the usual observation of a reciprocal relationship between guard-cell starch content and stomatal aperture size. For the lack of methods at the time, the theory was tested only semiquantitatively for starch and not at all for sugars. Upon the discovery that massive K^+ accumulation in guard cells accompanies stomatal opening and that K^+ loss from guard cells accompanies stomatal closure, the classical theory was discarded (for history, see Hsiao, 1976; Raschke, 1979; Outlaw, 1983). Subsequently, most biochemical studies of guard cells focused on the carbon metabolism associated with K^+ fluxes, such as the synthesis of malate. However, various potential roles for sugars remained (for summary, see Outlaw, 1983) primarily because occasional reports (e.g. Outlaw and Manchester, 1979) indicated that whole-cell Suc concentration and stomatal aperture size in guard cells are correlated, and other reports (e.g. MacRobbie and Lettau, 1980) indicated that K^+ alone is insufficient to cause the necessary $\Delta\Psi_s$.

A renewed interest in guard-cell carbon metabolism involving sugars was stimulated by two reports. First, Gotow et al. (1988) reported that sugar phosphates are formed by photosynthesis in guard cells of broad bean (*Vicia faba* L.). Second, Tallman and Zeiger (1988; see also Poffenroth et al., 1992; Talbott and Zeiger, 1993), also working with *V. faba*, reported that red light causes an increase in stomatal aperture size on epidermal peels and a decrease in guard-cell Ψ_s without either an increase in guard-cell K^+ concentration or a decrease in guard-cell starch content. Under other conditions, they found that stomata also open without an increase in guard-cell K^+ concentration but with a loss of guard-cell starch content. They indicated that their data are not consistent with K^+ being the universal guard-cell osmoticum, and they suggested, as additional osmotica, internal sugars arising from the PCRP or starch breakdown. Important to comparisons made with our work, they noted that stomata induced to open in intact leaves have a substantially higher K^+ content than those of epidermal peels discussed above. In summary, the work discussed above argues for two major revisions to the paradigm prevalent prior to 1988. First, a qualification to the role of K^+ as the sole osmoticum in stomatal movements is indicated. Second, a level of the PCRP in guard cells sufficient to contribute to the osmotic requirements of stomatal movements—which is not consistent with our interpretation of most quantitative investigations (Outlaw, 1989; Reckmann et al., 1990; Gautier et al., 1991)—is indicated.

Our work with stomata on attached leaves of *V. faba* led in a different direction. Hite et al. (1993) interpreted the high levels of guard-cell Suc synthase and of Suc-P synthase as indicating that guard cells are carbohydrate sinks,

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Abbreviations: $\Delta\Psi_s$, change in solute potential; PCRP, photosynthetic carbon reduction pathway; Ψ_s, solute potential.

not sources. Although we (Lu et al., 1995) found as before (Outlaw and Manchester, 1979) that the Suc concentration was higher in guard cells of open stomata than in guard cells of closed stomata, our surprising observation was that the guard-cell apoplast was the site of osmotically significant Suc accumulation in transpiring *V. faba* leaves. *V. faba* is an apoplastic phloem loader in which the apoplastic Suc concentration is elevated during photosynthesis (Delrot et al., 1983). Thus, we (Lu et al., 1995; Outlaw, 1995; Outlaw et al., 1996) hypothesized that the leaf apoplast (and, ultimately, mesophyll cells) was the source of the Suc that we observed at high concentrations in the guard-cell apoplast and that Suc may serve as a signal metabolite in the sensing of transpiration.

Here we tested these hypotheses by studying the kinetics of Suc-specific radioactivity in various leaf compartments following a pulse of $^{14}CO_2$. First, we found low ^{14}C contents in guard cells during the first 20 min after labeling, which eliminated guard-cell PCRP as a substantial source of the increase in Suc concentration in the guard-cell apoplast. Second, at 40 min after labeling, the Suc-specific radioactivity in the guard-cell apoplast was high, which eliminated starch breakdown as the substantial source of the increase in Suc concentration in the guard-cell apoplast. The results substantiate our view that there are previously unrecognized relationships among stomatal aperture size, photosynthesis, phloem loading, and transpiration.

MATERIALS AND METHODS

Broad bean (*Vicia faba* L. cv Longpod) was grown in a growth chamber in a potting medium (Metro-Mix 220, Scotts-Sierra Horticultural Products Co., Marysville, OH) at a density of two plants per 1-L pot. Illumination was supplied by fluorescent and incandescent lamps (wattage ratio [fluorescent to incandescent] approximately 6; maximum PAR at plant level approximately 600 μmol m^{-2} s^{-1}). Full, incandescent illumination began at 6 AM; beginning at 6:30 AM, fluorescent lamps were turned on at 30-min intervals in three equal lots. The lamps were turned off in steps, beginning at 9 PM, in a reverse of the illumination-onset sequence, except that the incandescent lamps were turned off with the last lot of fluorescent lamps. The dark-period temperature (20°C) was ramped up to the light-period temperature (25°C) during the illumination-onset sequence; similarly, the temperature was ramped back down during the light-to-dark transition. RH was constant at 60%. Plants were given supplemental liquid fertilizer (Peters 20-20-20, United Industries Corp., St. Louis, MO) at each watering. The youngest, fully expanded bifoliate was used in all of the experiments.

^{14}C-Labeling System

A ^{14}C-labeling system was assembled inside the plant-growth chamber, which minimized environmental perturbations to the plants. In brief, the closed system consisted of four Plexiglas chambers that were connected in a series: (a) A buffer chamber, in which $^{14}CO_2$ was generated by addition of 3 mL of 3 N $HClO_4$ to 18 μmol $Na_2{}^{14}CO_3$ (2000 GBq mol^{-1}). Inclusion of the buffer chamber was to increase the volume of the labeling system so that CO_2 concentration inside the system would be little affected by amendment to include $^{14}CO_2$ (+1.06 μM) or photosynthesis (−1 μM, estimated). Air was circulated (10 L min^{-1}) throughout the system by a squirrel-cage fan (Condor Industrial, Oxward, CA) at the inlet of this chamber. (b) The labeling chamber (11 [wide] × 48 [long] × 8.5 [high] cm), in which two baffles at the inlet created antechambers to mix further and dissipate turbulence of the air from the buffer chamber. The top of the labeling chamber was removed except during $^{14}CO_2$ labeling. Three 7-mm circular openings that were equally spaced along the long axis of the bottom of the chamber were for the separate insertion of three plants. (Insertion was facilitated by removal of one-half of the chamber bottom.) (c) A condensation chamber, which contained cool, water-filled coils onto which moisture condensed as the air temperature was lowered to 17°C to maintain the RH. (d) A reheat chamber, which contained warm, water-filled coils to reheat the air. At the end of the labeling period, the tubing leading into the labeling chamber was removed and the system air was exhausted through 3 N NaOH to trap the radioactive gas.

The growth chamber (air turnover ≥ 10 volumes h^{-1}, wind speed < 0.8 m s^{-1}) was housed in a large former animal-care facility with only outside make-up air (air-turnover ≥ 7 volumes h^{-1}). Photosynthetic rates were calculated using the dilution of the generated $^{14}CO_2$ into the chamber-system air (364 μL CO_2 L^{-1} and 764 mm Hg).

^{14}C Labeling and Sampling

Three hours after the onset of the photoperiod, three plants were trimmed by removing the shoot 3 mm above the experimental leaf. Then, the plants just below the experimental leaves were sealed individually through one of three openings in the bottom of the labeling chamber. After an additional 2 h, the chamber top was added and the leaves (each comprising a pair of bifoliates) were labeled for 5 min. At the end of the $^{14}CO_2$ pulse, the chamber top was removed and leaves were harvested at the indicated times (Figs. 1–6). Because the labeling chamber accepted only three plants, each experiment required two labeling episodes. Three samples from each leaf were taken in the following order: (a) A 2- × 2-cm square, free of major veins, was cut from one side of one leaflet. This tissue was frozen immediately in liquid nitrogen for subsequent histochemical analysis ("palisade cells" and "guard cells"). (b) Within 1 min, an abaxial epidermal peel from the opposite side of this same leaflet was taken and floated mesophyll-side down on excess water (100 mL) for 2 min to remove apoplast solutes; then, it also was frozen immediately for histochemical analysis ("guard-cell symplast"). (c) The sister leaflet was placed in a pressure chamber (model 100, PMS Instrument, Corvallis, OH), which was fitted with a custom seal (M.S. Ewert and W.H. Outlaw Jr., unpublished data) that minimized damage to the petiole. The sap first expressed and possibly contaminated by broken-cell contents (about 3 μL) was blotted away and discarded. The next 3 μL of sap expressed was collected for subsequent assay.

One experiment is shown in the figures to show pairwise comparisons between palisade cells and guard cells of the same leaflet. Derivative data (ratios of ^{14}C in guard cells compared with palisade cells) show the relationship without a confounding effect of differences in CO_2 incorporation rates among leaflets. A replicate experiment, as well as unpublished experiments, gave essentially the same results, as mentioned in the "Results."

Histochemical Procedures

General, quantitative histochemical procedures (Passonneau and Lowry, 1993) were used to obtain stable, single-cell samples of palisade cells, of guard cells, and of guard cells devoid of apoplastic solutes (for dissection precision, see Hampp and Outlaw, 1987). The oil-well technique was used for extractions and for relevant microprocedures. Different subsamples of leaflets and of epidermal peels for the displayed experiment were assayed (compare with Outlaw and Manchester, 1979) to ensure that aberrant cells did not result in erroneous conclusions.

Suc Assays

The assay of Jones et al. (1977), over the range of 0.1 to 10 pmol, was used except that *N,N*-bis[2-hydroxyethyl]-2-aminoethane-sulfonic acid was substituted for imidazole.

^{14}C and [^{14}C]Suc Assays

Pooled cells (either 10 palisade cells or 20 guard cells per sample) were extracted in 10 μL of water under oil in an oil-well rack that had been preheated to 95°C. The oil-well rack was immediately returned to 95°C for 30 min. One aliquot (2.8 μL) was used directly for the ^{14}C assay. A second aliquot (0.5 μL) was used for Suc analysis. A third aliquot (5.7 μL) was mixed with 20 mg of mixed-bed ion-exchange resins (TMD-8, Sigma) to remove potentially radioactive organic ions. Then, the neutral supernatant was amended to include 0.1 mM authentic Suc and subsequently fractionated by HPLC (4- × 250-mm column, PA1, CarboPac, Dionex, Sunnyvale, CA, with 150 mM NaOH mobile phase at a flow rate of 0.8 mL min^{-1}). Authentic Suc elution, which indicated the position of the [^{14}C]Suc of the extract, was identified by a pulsed amperometric chromatographic detector.

The ^{14}C assay was done by liquid scintillation counting with special precautions because of the limited amount of ^{14}C present (e.g. multiple extractions for each sample using cells from different leaf fragments, multiple counting periods interspersed with background counts for each sample, background counts of each vial before sample addition, and internal standards).

RESULTS

Suc-Specific Radioactivities and Pool Sizes in Palisade Cells and in Petiolar Sap following a Pulse of $^{14}CO_2$ to Intact Leaves

On the basis of acid-stable ^{14}C present at the end of the $^{14}CO_2$ pulse, we calculated that palisade cells (data not shown) had a rate of ^{14}C incorporation (soluble fraction) of approximately 14 ± 5 pmol CO_2 $cell^{-1}$ h^{-1} (mean ± SE). This rate of incorporation is equivalent to 86 μmol CO_2 mg^{-1} chlorophyll h^{-1} (conversion factors from Outlaw et al., 1985), which may be compared with a reference value of 20 to 60 μmol CO_2 mg^{-1} chlorophyll h^{-1} for enzymically isolated mesophyll cells of *V. faba* (Outlaw et al., 1976).

The Suc-specific radioactivities of palisade cells of different leaflets harvested at various times during the chase period followed a regular time course (Fig. 1). Initially, the Suc-specific radioactivity was low and remained so for 15 min, after which it increased to peak at a value of 111 GBq mol^{-1} (20-min point, Fig. 1). Following this peak, the Suc-specific radioactivity decreased slowly over the next 40 min to a minimum value, approximately 10% of its peak value.

During the course of the experiment, the palisade Suc pool size was at a steady state (2.6–3.3 pmol $cell^{-1}$). The absolute values in the present experiments were consistent with earlier estimates (Jones et al., 1977; Outlaw and Manchester, 1979).

The maximum Suc-specific radioactivity of petiolar sap (0.54 GBq mol^{-1}, Fig. 2) occurred at 15 min, i.e. this maximum preceded and was approximately 200-fold less than that of whole palisade cells (Fig. 1).

The Suc concentration of petiolar sap of these photosynthesizing shoot-pruned plants was at a steady state (in the

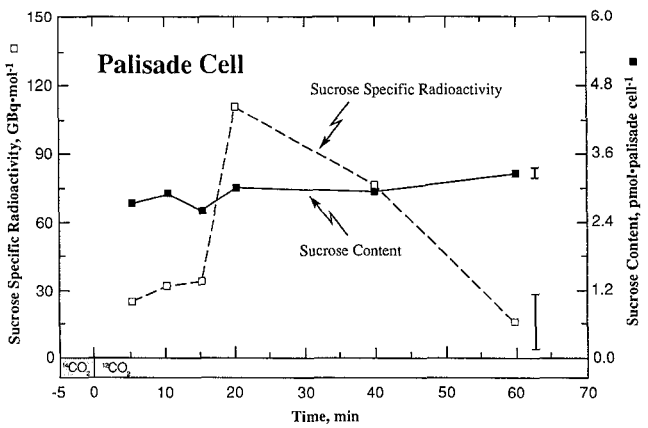

Figure 1. The Suc-specific radioactivity (□) and Suc content (■) of palisade-parenchyma cells in different leaflets at indicated times following a 5-min pulse of $^{14}CO_2$. Intact, attached leaflets of *V. faba* L. were labeled under daytime growth conditions (briefly, 25°C, 60% RH, 600 μmol photons m^{-2} s^{-1}, and 16 μmol CO_2 L^{-1}; see "Materials and Methods"). At the indicated times, a leaflet was quick-frozen and freeze-dried. Extracts of pooled samples of individually dissected palisade cells were assayed for Suc using quantitative histochemical methods. Other aliquots of these extracts were fractionated by HPLC, and the fractions containing Suc were assayed for radioactivity. The error bar associated with Suc content is the largest SE for triplicate Suc assays. The error bar associated with Suc-specific radioactivity is the 95% confidence interval of the radioactivity assays calculated according to an algorithm resident in the spectrometer. The displayed results are similar to other results obtained with different samples taken from the same leaflets (indicating the absence of significant intraleaflet variation) and to those of a replicate experiment. The six leaflets used to obtain the data for these six times were also used for dissection of other displayed cell data (Figs. 3–6).

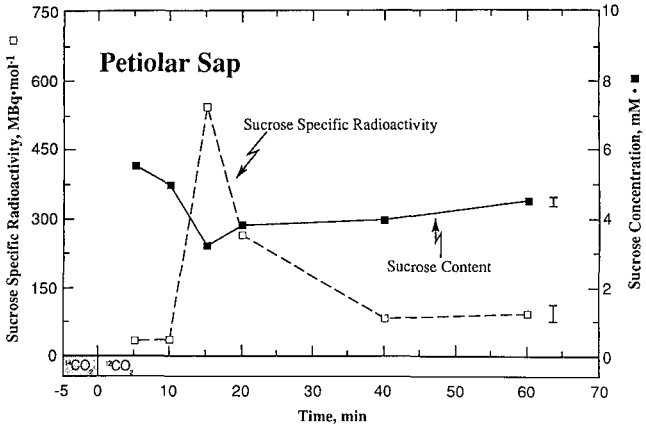

Figure 2. The Suc-specific radioactivity (□) and Suc content (■) of petiolar sap expressed at indicated times from a $^{14}CO_2$ pulse-labeled leaflet. The leaflets used for sap collection were the sister leaflets of those used for cell data (Figs. 1 and 3–6). The error is the largest SE for replicate radioactive assays (n = 7); essentially no error was associated with the Suc assay. For other details, see Figure 1.

range of 3.1–5.5 mM, Fig. 2). Preliminary experiments (not shown) indicated that shoot pruning did not have a confounding effect on the Suc concentration in petiolar sap. Literature values for apoplastic Suc concentration vary considerably, but our value is typical (Lohaus et al., 1995).

The Kinetics of ^{14}C Content of Guard Cells following a Pulse of $^{14}CO_2$ to Intact Leaves

The apparent time course for the soluble ^{14}C content of guard cells was determined by assaying extracts of cells dissected from leaflet fragments harvested during the postlabeling period (Fig. 3). (Guard cells dissected from leaflet fragments contain both the symplastic and apoplastic pools.) During the first 20 min of the postlabeling period, guard cells contained relatively little soluble ^{14}C. On an absolute basis, the average ^{14}C contents of the first three guard-cell samples (up to 15 min, Fig. 3) were equivalent to an estimated C incorporation rate of 0.7 ± 0.2 pmol $cell^{-1}$ h^{-1}.

The ^{14}C content of guard cells in situ increased dramatically between 20 and 40 min ($[^{14}C_{guard\ cell}]/^{14}C_{palisade}$] = 0.04 at 20 min and 0.8 at 40 min, Fig. 3, and in the replicate experiment, 0.07 and 0.4, respectively). On an absolute basis, guard cells of the leaflet harvested at 40 min contained 3.4-fold ^{14}C, as did those of the leaf harvested at 20 min.

Suc-Specific Radioactivities and Pool Sizes in Guard Cells following a Pulse of $^{14}CO_2$ to Intact Leaves

Figure 4 shows the apparent time course of guard-cell Suc-specific radioactivity. The data paralleled those of the ^{14}C content (Fig. 3) and, thus, implied that [^{14}C]Suc, or a precursor, was imported into guard cells. The guard-cell Suc-specific radioactivity maximum (154 GBq mol^{-1}, 40 min, Fig. 4), exceeded that of palisade-cell Suc-specific radioactivity (111 GBq mol^{-1}, 20 min, Fig. 1), an observation that will be interpreted (see "Discussion") in the context of two palisade-cell Suc pools and inhomogeneity in the leaf apoplast. The same trend was observed in the replicate experiment (the guard-cell Suc-specific radioactivity was only 0.07-fold that of palisade cells at 20 min, but the ratio increased to 5.4 at 40 min).

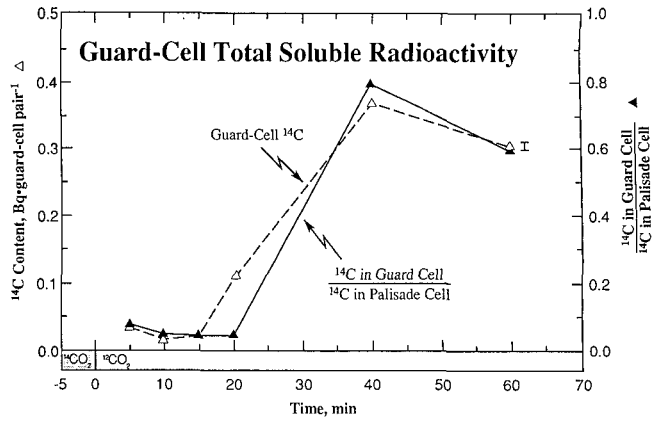

Figure 3. The water-soluble ^{14}C content of guard cells (△) individually excised from pulse-labeled intact leaflets, and the instantaneous ratio of the guard-cell water-soluble ^{14}C content to that in palisade cells (▲). On the basis of the ^{14}C in the 5-, 10-, and 15-min points and the specific radioactivity of the $^{14}CO_2$ used, the ^{14}C incorporation rate estimate was 0.7 pmol guard $cell^{-1}$ h^{-1}. This rate estimate is imprecise because of the percentage counting error in determining the low disintegrations per minute in the initial guard-cell samples. The main value of this estimate is its use as a benchmark for a comparison with the ^{14}C incorporation rate of palisade cells (14 pmol $cell^{-1}$ h^{-1} ≈ 86 μmol mg^{-1} chlorophyll h^{-1}). The error bar is the largest SE for replicate radioactive assays (n = 6). For other details, see Figure 1. Each point for all displayed data for guard cells (Figs. 3–6) was replicated with guard cells dissected from more than one area of the sampled leaflets; in addition, data from a replicate experiment were corroborative.

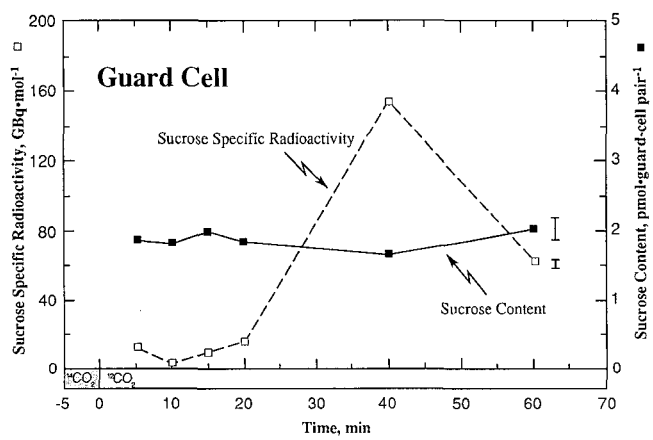

Figure 4. The Suc-specific radioactivity (□) and Suc content (■) of guard cells in different leaflets at indicated times following a 5-min pulse of $^{14}CO_2$. These cells, dissected from an intact leaflet, contained both the symplastic and apoplastic Suc compartments. The error associated with Suc-specific radioactivity is the largest SE for replicate radioactive assays (n = 7). For other details, see Figures 1 and 3.

The Suc content of whole guard cells (Fig. 4) was approximately 2 pmol guard-cell pair^{-1} (equivalent to 330 mmol kg^{-1} dry mass; compare with Lu et al., 1995) and was 2-fold more than the concentration of Suc in guard cells of leaves under low-CO_2 conditions (Outlaw and Manchester, 1979).

[^{14}C]Suc Pools of the Guard-Cell Symplast and Guard-Cell Apoplast following a Pulse of $^{14}CO_2$ to Intact Leaves

The apparent time course for guard-cell symplastic Suc-specific radioactivity was determined by assaying extracts of cells dissected from rinsed epidermal peels harvested during the postlabeling period (Fig. 5A). As found for the whole cells, the initial levels of radioactivity were low. Thus, these initial values (Fig. 5A) lack precision, and we do not interpret the fluctuations in guard-cell symplastic Suc-specific radioactivities during the first 15 min of the postlabeling period (Fig. 5) to have biological significance. For the 20-min postlabeling sample, in which the palisade-cell Suc-specific radioactivity was maximum, guard-cell symplastic Suc-specific radioactivity remained low, 0.3-fold that of palisade cells (Fig. 5A; 0.24-fold in the replicate experiment). The guard-cell symplastic Suc-specific radioactivity of the 40-min postlabeling sample was dramatically higher than that at 15 or 20 min postlabeling (Fig. 5A), mimicking the pattern for the total soluble ^{14}C. More significantly, and a major element of this report, the ratio of guard-cell symplastic Suc-specific radioactivity to palisade-cell Suc-specific radioactivity increased to 1.2 at 40 min postlabeling (Fig. 5B; 1.4 in the replicate experiment) and to 3.6 at 60 min postlabeling. Whereas this ratio of instantaneous values exceeded unity, the ratio based on the maximum value of the palisade-cell Suc-specific radioactivity did not achieve unity (Fig. 5B).

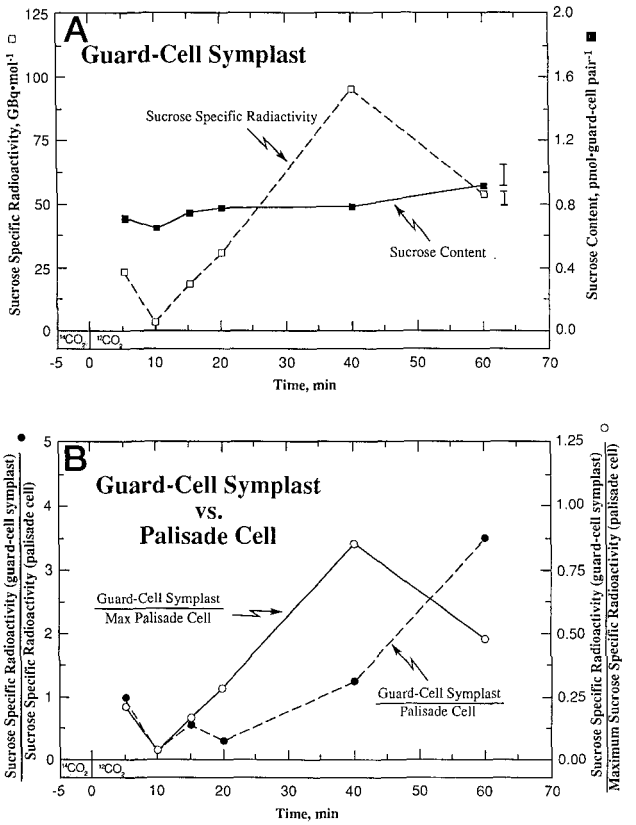

Figure 5. A, The Suc-specific radioactivity (□) and Suc content (■) of the guard-cell symplast in different leaflets at indicated times following a 5-min pulse of $^{14}CO_2$. These values were obtained from guard cells dissected from a rinsed epidermal peel taken at the indicated time from an intact leaflet. The error associated with Suc-specific radioactivity is the largest SE for replicate radioactive assays (n = 7). B, The ratio of the guard-cell symplast Suc-specific radioactivity (A) divided by the palisade-cell Suc-specific radioactivity (Fig. 1) following a 5-min pulse of $^{14}CO_2$ to an intact leaflet. One trace (●) is the instantaneous ratio and the other trace (○) is the ratio using the maximum palisade-cell Suc-specific radioactivity (20 min, Fig. 1). For other details, see Figures 1 and 3.

The Suc content of the guard-cell symplast (Fig. 5A) was 0.65 to 0.9 pmol guard-cell pair^{-1} and seemed to increase over the course of the experiment (r^2 = 0.76). As discussed earlier (Lu et al., 1995), the guard-cell symplastic Suc concentration under the present conditions did increase from dawn to midday, but the osmolality change was relatively minor in the context of stomatal movements, unless Suc accumulation was restricted to a small region of the symplast.

The apparent time course for guard-cell apoplastic Suc-specific radioactivity (Fig. 6) was constructed from data for the whole cell (Fig. 4) and for the guard-cell symplast (Fig. 5A). The guard-cell apoplastic Suc-specific radioactivity increased remarkably during the period 20 to 40 min postlabeling to 204 GBq mol^{-1} (Fig. 6A), which was higher than that of guard cells (154 GBq mol^{-1}, 40 min, Fig. 4) and that of whole palisade cells (111 GBq mol^{-1}, 20 min, Fig. 1). In the replicate experiment, [^{14}C]Suc was not detectable at 20 min in the guard-cell apoplast, but at 40 min, the Suc-specific radioactivity in the guard-cell apoplast was 8.4-fold that of the palisade cells.

The Suc content of the guard-cell apoplast (Fig. 6) was 0.9 to 1.3 pmol guard-cell pair^{-1} and appeared to be constant over the course of the experiment. The guard-cell-wall volume of *V. faba* is 8.3 ± 0.3 pL stoma^{-1} (S.Q. Zhang and W.H. Outlaw Jr., unpublished data).

DISCUSSION

In Planta Rates of ^{14}C Incorporation by Palisade Cells and Guard Cells Are Vastly Different

The rate of CO_2 incorporation by the palisade cells was approximately 14 pmol CO_2 cell^{-1} h^{-1}. Conversion of this rate to photosynthesis on a cell or leaf area basis (Fig. 7) provides a quantitative perspective as a C input for a compartmental analysis. Thus, on a cell-volume basis, the rate of photosynthesis in *V. faba* palisade cells is approximately 2-fold that of spongy cells at the experimental PAR (Outlaw and Fisher, 1975b), but the protein-to-chlorophyll ratio in palisade cells is approximately 0.5-fold that of spongy cells (Outlaw et al., 1976). Our method of conversion (86 μmol CO_2 mg^{-1} chlorophyll h^{-1} and 27 mg fresh leaf cm^{-2} leaf area, S.Q. Zhang and W.H. Outlaw Jr., unpublished data; a nominal protein content of 2%, and a nominal protein to chlorophyll ratio of 35) tended to cancel these differences

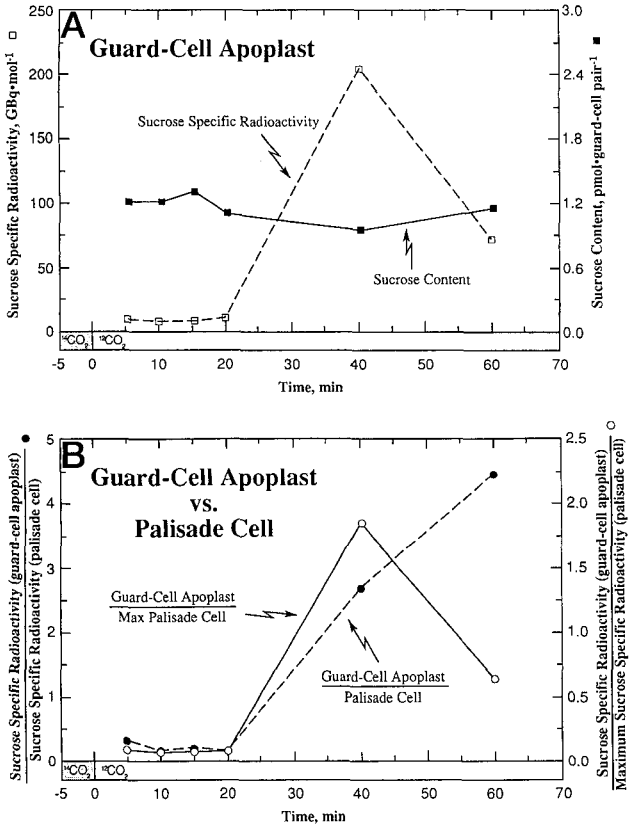

Figure 6. A, The Suc-specific radioactivity (□) and Suc content (■) of the guard-cell apoplast in different leaflets at indicated times following a 5-min pulse of $^{14}CO_2$. These values were calculated from whole-cell data (Fig. 4) and symplastic data (Fig. 5A). B, The ratio of the guard-cell apoplast Suc-specific radioactivity (Fig. 6A) divided by the palisade-cell Suc-specific radioactivity (Fig. 1) following a 5-min pulse of $^{14}CO_2$ to an intact leaflet. One trace (●) is the instantaneous ratio and the other trace (○) is the ratio using the maximum palisade-cell Suc-specific radioactivity (20 min, Fig. 1). For other details, see Figures 1 and 3.

and yielded an estimate for the overall mesophyll photosynthesis of 1.3 μmol CO_2 cm^{-2} leaf area h^{-1}.

On a cell basis, guard cells incorporated only 5% as much ^{14}C as did palisade cells (not shown). On a leaf area basis, the rate of $^{14}CO_2$ incorporation was 0.013 μmol CO_2 cm^{-2} leaf area h^{-1} (conversion factors in Outlaw, 1983). Whereas we caution that the individual values for ^{14}C incorporation by guard cells were imprecise because of the low levels of ^{14}C in the initial samples, in aggregate the rates are in the range of organic anion synthesis in planta in guard cells (guard-cell malate accumulation rate: 0.33 pmol $cell^{-1}$ h^{-1}, Outlaw and Kennedy, 1978; guard-cell PEP carboxylase activity: 3–4.5 pmol $cell^{-1}$ h^{-1}, Tarczynski and Outlaw, 1990; Wang et al., 1994). Corroboratively, on a cell basis, the total amount of [^{14}C]Suc in palisade parenchyma was approximately 30-fold that of the guard-cell symplast at 20 min (332 versus 12 mBq $cell^{-1}$, Fig. 7). As will be discussed later, guard cells imported relatively large quantities of ^{14}C (Figs. 3 and 7), and the contribution of guard-cell CO_2 reduction, if any, to their metabolism was insignificant when compared with the ^{14}C that they imported.

[^{14}C]Suc in the Palisade Parenchyma Provides a Basis for Compartmental Analysis

Earlier, we (Outlaw et al., 1975; Fisher and Outlaw, 1979) reported that Suc-specific radioactivities in the whole leaflet, in palisade parenchyma cells, in upper spongy parenchyma cells, and in lower spongy parenchyma cells peak 15 to 20 min after briefly pulse-labeling *V. faba* leaflets with $^{14}CO_2$. Because the kinetics of Suc-specific radioactivities in the three mesophyll layers are qualitatively similar (Outlaw et al., 1975), for this study we assayed only palisade cells. We favored the use of palisade cells because they are easiest to select uniformly, they have the highest radioactivity after a pulse of $^{14}CO_2$ (Outlaw and Fisher, 1975a, 1975b), and we (Fisher and Outlaw, 1979) have studied them more intensively. To obtain the original data, we (Outlaw et al., 1975; Fisher and Outlaw, 1979) pulse-labeled a single leaflet and, at various times during the ^{12}C chase, leaf punches were taken for measurement of Suc-specific

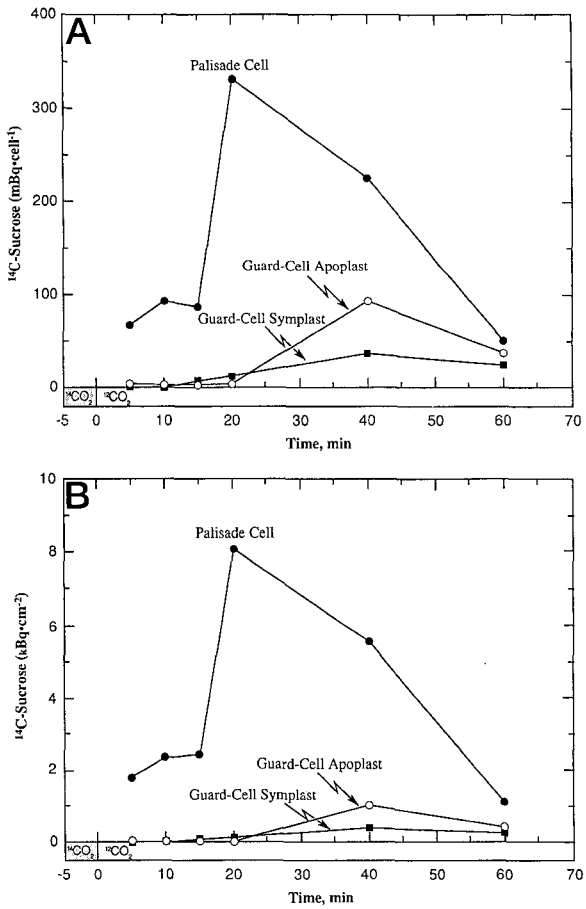

Figure 7. A, Kinetics of the total [^{14}C]Suc pools on a cell basis in the palisade cell (●), guard-cell symplast (■), and guard-cell apoplast (○) following a pulse of $^{14}CO_2$. B, Kinetics of the total [^{14}C]Suc pools on a tissue basis in the palisade cell (●), guard-cell symplast (■), and guard-cell apoplast (○) following a pulse of $^{14}CO_2$. The data for guard cells were calculated for only the abaxial epidermis.

radioactivities of different Suc pools. Thus, a leaflet served as its own internal control for the rate of ^{14}C incorporation (Outlaw et al., 1975). Replicates of this experiment formed the basis for construction of a compartmental model. An adaptation (Outlaw, 1995) of that model (Fig. 8) and use of [^{14}C]Suc in palisade parenchyma (Fig. 7) as a quantitative benchmark allows us to interpret the present results, as described in the following sections.

The present experiments differed from the original ones in two ways. First, the light intensity was higher, which would alter the relative input coefficients into the different mesophyll [^{14}C]Suc precursor pools, as discussed in the previous section (Fig. 8). Second, the protocol was not the same as that used before. However, the Suc-specific radioactivities of palisade cells of different leaflets harvested at various times during the chase period as reported here (Fig. 1) fit the composite kinetics data that we modeled (Fig. 8; Outlaw et al., 1975; Fisher and Outlaw, 1979). Thus, it was unnecessary to normalize the data to account for the differences in ^{14}C incorporation among leaflets, and we used the raw results (Fig. 1) as a quantitative reference (Fig. 7). As a validation of our procedures, we also presented the results (Figs. 3–6) as ratios of radioactivities and as ratios of Suc-specific radioactivities in different pools within the same leaf (compare with Outlaw and Fisher, 1975a, 1975b; Outlaw et al., 1975).

The Suc-specific radioactivities for whole palisade cells (Figs. 1 and 8) derive from two major Suc pools: a mobile pool originally identified as the cytosol and a relatively nonmobile pool, the vacuole (Fisher and Outlaw, 1979). Compared with the vacuolar pool, the cytosolic pool reaches a higher Suc-specific radioactivity, reaches the maximum Suc-specific radioactivity earlier, turns over more rapidly, and is smaller (Fisher and Outlaw, 1979). Applied to the present data (Fig. 1), our model (Fig. 8) predicts that the Suc-specific radioactivity in the palisade cytosol would have peaked at approximately 8 min with a maximum value of 353 GBq mol^{-1}. The peak in the Suc-specific radioactivity in the veins would have reached a maximum at 18 min; an estimate for this value (220 GBq mol^{-1}) is predicted by the original model. Together, these predictions indicate that the Suc-specific radioactivity in the apoplast near the veins would have reached a maximum of 220 to 353 GBq mol^{-1} at 8 to 18 min after pulse-labeling.

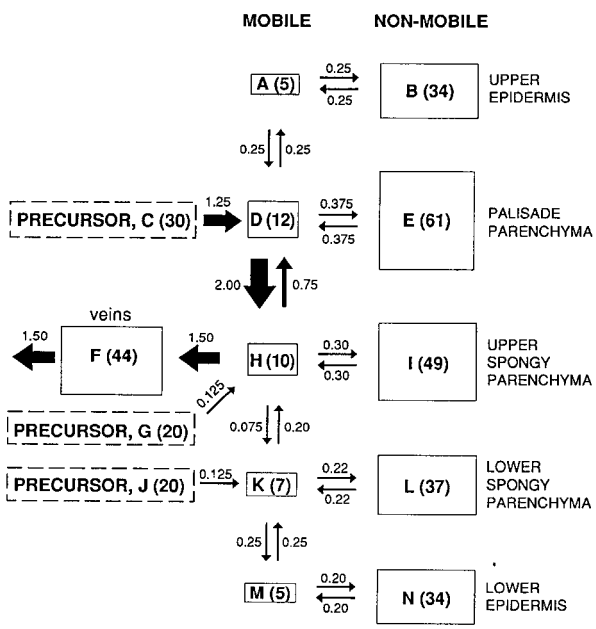

Figure 8. An adaptation (Outlaw, 1995) of a model (Outlaw et al., 1975) that describes Suc compartmentation in the *V. faba* leaflet. The sizes of and numbers by the arrows represent relative exchange coefficients with inputs obtained at relatively low light intensity (Outlaw and Fisher, 1975a; Outlaw et al., 1975). At the higher light intensities of the work reported in this paper, the inputs into the mobile pools of the spongy parenchyma would be higher (Outlaw and Fisher, 1975b). The sizes of and the numbers within each pool are proportional to pool size (nmol cm^{-2}).

The Leaf Apoplast Is Not a Single Homogeneous Well-Mixed Suc Pool

As expected (Fig. 8), the time course for the peak in Suc-specific radioactivity of the apoplast measured as petiolar sap (Fig. 2) fell between the predicted peaks of [^{14}C]Suc in the palisade-parenchyma cytosol and the veins. However, the absolute maximum value of the Suc-specific radioactivity of petiolar sap was more than 2 orders of magnitude smaller than either. Because Suc in the apoplast is an intermediate between these other two compartments (Bouché-Pillon et al., 1994), this difference is a first line of evidence that Suc in the apoplast is not a single, rapidly mixed homogeneous pool. Second, a calculation made from the apoplast volume (6.6 μL cm^{-2}, pressure-bomb method, M.S. Ewert, S.Q. Zhang, W.H. Outlaw Jr., unpublished data) the Suc concentration in the petiolar sap (Fig. 2), and the total amount of $^{14}CO_2$ incorporation by the mesophyll yields an extreme upper-limit estimate of the average apoplastic Suc-specific radioactivity (95–154 GBq mol^{-1}). Again, this value is less than that of veins, indicating that they must be surrounded by a localized apoplastic Suc pool. Finally, prima facia evidence—a comparison of the Suc specific radioactivities in the petiolar sap (Fig. 2) and the guard-cell apoplast (Fig. 6A)—is a direct indication of apoplastic heterogeneity.

Suc in the Guard-Cell Apoplast Is Derived from the Mesophyll and Turns Over Rapidly

The kinetics of Suc-specific radioactivities in palisade cells (Figs. 1 and 7), the guard-cell symplast (Fig. 5), and the guard-cell apoplast (Fig. 6), along with the total pool sizes of ^{14}C in these compartments, indicate that [^{14}C]Suc excreted from the mobile Suc pool in the mesophyll moves in the apoplastic solution to the terminal point in the evaporative pathway, the guard-cell wall. As established earlier, the predicted peak in the apoplastic Suc-specific radioactivity should be highest around the mesophyll cells (peak at 8–18 min, 220–353 GBq mol^{-1}), which is consistent with this pool being a donor to the guard-cell apoplast (peak at 40 min, 204 GBq mol^{-1}).

Approximately 30 min separated the predicted peak of the Suc-specific radioactivity in the mobile palisade pool and the measured peak of Suc-specific radioactivity in the guard-cell apoplastic pool. During this time transpirational water loss was 3.1 μL cm^{-2} (calculated from conductance). Use of this volume for dilution of [^{14}C]Suc upon secretion from the mesophyll indicates a maximum Suc-specific radioactivity of 202 to 327 GBq mol^{-1}. The Suc-specific activity of the localized volume is consistent with its being a donor to the guard-cell apoplast.

An assumption that import of ^{14}C to the guard-cell apoplast did not occur after 40 min permitted us to treat the loss of ^{14}C from the guard-cell apoplast as a first-order process with a kinetic constant that was used to calculate a Suc efflux rate of 0.05 pmol stoma^{-1} min^{-1}. This rapid loss of Suc from the guard-cell apoplast indicates that this pool size would decrease rapidly in the absence of a high input rate (compare with Lu et al., 1995).

Guard Cells Import Mesophyll-Derived Suc from the Apoplast

Considered together, the kinetics of total soluble ^{14}C in guard cells (Fig. 3) and the kinetics of Suc-specific radioactivities in the palisade cells (Figs. 1 and 7) and in guard cells (Figs. 4–7B) clearly imply that guard cells import ^{14}C from the mesophyll, the only tissue that is capable of high rates of $^{14}CO_2$ fixation.

The kinetics did not permit an exact calculation of the rate at which [^{14}C]Suc was imported into guard cells. However, we calculated a lower limit (12–21 fmol min^{-1} stoma^{-1}) by two methods, net influx and exponential decline. As a perspective in the context of membrane transport, this "physiological" lower limit is 3- to 6-fold more than in vitro rates for *V. faba* guard cells with 30 mM external Suc (Outlaw, 1995). The higher in planta rates are consistent with a high concentration of Suc in the guard-cell apoplast. On a membrane-area basis, this lower-limit estimate (up to 10.6 pmol Suc cm^{-2} guard-cell surface area s^{-1}) is approximately the same as the rate of K^+ accumulation during stomatal opening (Outlaw, 1983). Because a mechanism for plant-cell Suc uptake is by H^+-symport (Bush, 1993), Suc uptake would be depolarizing and, thus, oppose stomatal opening (for mechanisms, see Schroeder et al., 1994; Outlaw et al., 1996). At present, however, it is not possible to establish the quantitative significance of competition between K^+ uptake and Suc uptake by guard cells.

We note that our calculated lower limit for guard-cell Suc uptake would be sufficient to replenish in 20 min the starch degraded during stomatal opening (Outlaw and Manchester, 1979) or exceed respiration by 15-fold (median of literature values collected in table II, Gautier et al., 1991).

Suc in the Guard-Cell Apoplast Has a Significant Osmotic Effect

Assuming 0.4 mL cm^{-3} to be a reasonable value for the water-free space-to-cell-wall volume ratio, we estimated the water-free space volume of *V. faba* guard-cell walls to be 3.3 pL stoma^{-1}. After conversion of the Suc content (midday, present experiments and those of Lu et al., 1995) to a molality basis (0.6 mL g^{-1}, solute-volume displacement) and correction for the variable osmotic coefficient of Suc (Michel, 1972), we calculated that Ψ_s(Suc) in the guard-cell wall changed by −1.0 MPa from dawn to midday. The $\Delta\Psi_s/\Delta\mu$m aperture relationships of Fischer (1972) and Raschke (1979) for *V. faba* indicate that decreasing the water potential by 1.0 MPa would depress stomatal aperture size by 3 μm. We consider this effect to be a reasonable estimate and one that may be improved by further work.

We briefly note the possibility of other effects of Suc accumulation in the guard-cell apoplast (e.g. gene expression) and that the mechanism that deposits Suc in the guard-cell apoplast would deposit other apoplastic solutes (e.g. malate or ABA) there, too.

Hypotheses: Suc in the Guard-Cell Wall Is a Physiological Signal That Integrates the Rates of Transpiration, Photosynthesis, and Translocation

Our summary interpretation is presented in Figure 9. Apoplastic fluid moves into the leaf by bulk flow in large tracheary elements (Canny, 1995). Recent mathematical considerations (Canny, 1995) point to diffusional movement in smaller tracheary elements, which is consistent with a need to avoid a transpirational flux in the minor veins that would wash Suc away from the site of phloem loading (Van Bel, 1993). Further movement of fluid, through the porous walls of mesophyll cells, may be by diffusion (Tyree and Yianoulis, 1980) or by bulk flow along the surfaces of hydrated walls. It is generally believed that most evaporation of the transpiration stream occurs in or

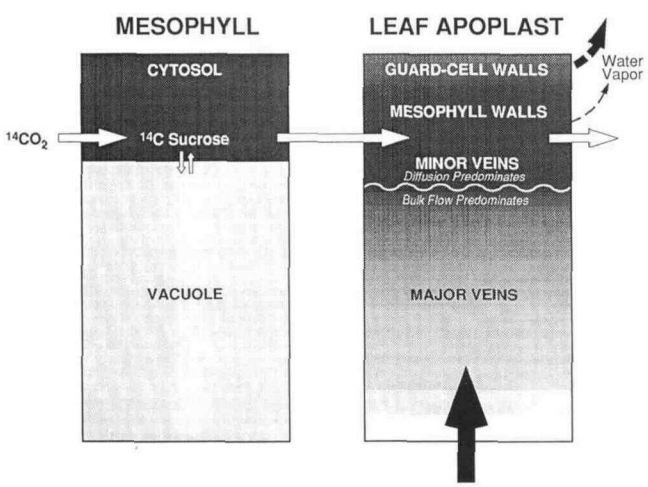

Figure 9. Mesophyll-derived Suc accumulates in the guard-cell walls of open stomata because of the chromatography effect (Tyree and Yianoulis, 1980; solutes accumulate at the distal point in an evaporative pathway) and peristomatal evaporation (Maier-Maercker, 1983; evaporation from or near guard cells). This model, with the darker regions representing pools rich in recently synthesized Suc, indicates that high transpiration rates (Mott and Parkhurst, 1991) or elevated Suc concentration in the apoplast would enhance Suc accumulation in guard-cell walls and, thus, attenuate stomatal aperture size.

near the guard-cell walls (Tyree and Yianoulis, 1980; Pickard, 1982; Maier-Maercker, 1983; Yianoulis and Tyree, 1984; but see also the discussion of Boyer, 1985). In any case, Suc would accumulate at the distal point in the pathway, the guard-cell wall, because of the chromatography effect (Tyree and Yianoulis, 1980). The accumulation of Suc in the guard-cell wall would increase with two factors: (a) the Suc concentration in the apoplast and (b) the rate of transpiration. The first factor, Suc concentration, is the net effect of Suc release from mesophyll (related to current photosynthesis and, perhaps, light [for refs., see Van Bel, 1993]) and efficiency of transport from the leaf (demonstrated by blockage of translocation [Ntsika and Delrot, 1986; Lohaus et al., 1995, and refs. therein]). Thus, our first hypothesis is that high apoplastic Suc concentration is a signal that causes a diminution of stomatal aperture size. The second factor, transpiration rate, has been elegantly shown (Mott and Parkhurst, 1991) to be a means by which plants respond to humidity (also see interpretations of Monteith, 1995). Thus, our second hypothesis is that accumulation of Suc in the guard-cell wall is a mechanism for attenuating conductance at high transpiration rates.

With implicit reservations, we wish to try to place the proposed mechanism in the context of optimizing CO_2 uptake by the plant. There is an approximately linear empirical relationship between stomatal aperture size and conductance, at least in the mid-aperture size range (for several species, see Waggoner and Zelitch, 1965; Weyers and Meidner, 1990). This empirical relationship has a theoretical foundation (equation 8.5, Nobel, 1983): stomatal conductance is inversely proportional to the mean pore "radius" ($\equiv [(\text{pore area})/\pi]^{0.5}$). Transpiration and conductance are linearly related, with the proportionality factor being the driving force for water movement. Thus, as a broad generality, there is a linear relationship between stomatal aperture size and water loss from the plant. With exceptions, the CO_2 assimilation rate plotted against conductance defines a rectangular hyperbola (Raschke, 1976; Farquhar and Sharkey, 1982) so that the increase in assimilation above approximately $g = 0.3$ mol m^{-2} s^{-1} is small. In summary, the plant becomes more water-use efficient at lower conductances, i.e. in general, at smaller stomatal aperture sizes. Thus, a low transpiration rate, even at high conductances, would not cause stomatal aperture size attenuation by this mechanism and photosynthesis could proceed at near maximum rates. Conversely, when photosynthetic Suc secretion into the leaf apoplast exceeds the leaf's capacity for removal, accumulation of Suc in the guard-cell apoplast would attenuate pore size and cause an increase in water-use efficiency with only a minor reduction in photosynthetic rate.

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