Running Head: Mesophyll-derived sucrose in the guard-cell apoplast

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DISCLAIMER

Title: A new mechanism for the regulation of stomatal-aperture size in intact leaves: accumulation of mesophyll-derived sucrose in the guard-cell wall of *Vicia faba* L.[1]

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ABSTRACT

At various times after pulse labeling *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 cytoplastic contents. Sucrose specific radioactivity peaked in palisade cells, 111 GBq·mol^{-1}, at 20 min. In contrast, the ^{14}C content and sucrose 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 sucrose specific radioactivity (204 GBq·mol^{-1}) and a high sucrose influx rate (0.05 pmol ·stoma^{-1}·min^{-1}). These and other comparisons implied the presence of (a) multiple sucrose pools in mesophyll cells, (b) a localized mesophyll-apoplast region that exchanges with phloem and stomata, and (c) mesophyll-derived sucrose in guard-cell walls sufficient to diminish stomatal opening by ~ 4 μm. Factors expected to enhance sucrose accumulation in guard-cell walls are (a) high transpiration rate, which closes stomata, and (b) high apoplastic sucrose concentration, which is elevated when mesophyll-sucrose efflux exceeds translocation. Therefore, multiple physiological factors are integrated in the attenuation of stomatal-aperture size by this previously unrecognized mechanism.

INTRODUCTION

For many years, sugars played a prominent role in 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 lack of methods at the time, the theory was tested only semi-quantitatively 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 on guard cells focused on the carbon metabolism associated with K^+ fluxes, such as the synthesis of malate. However, various potential roles for sugars remained (summarized by Outlaw, 1983) primarily because occasional reports (e.g., Outlaw and Manchester, 1979) indicated that whole-cell guard-cell suc concentration and stomatal-aperture size 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) observed that sugar phosphates are formed by photosynthesis in guard cells of *Vicia faba*. Second, Tallman and Zeiger (1988), 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. On the basis of results obtained from sonicated epidermal peels using a combination of red and blue light at different intensities, Poffenroth et al. (1992) concluded that internal soluble sugars (up to 550 fmol·guard $cell^{-1}$, ~ 100-150 mM, 91% suc) contribute significantly to the osmoregulation of guard cells in detached epidermes of *V. faba*. Consistent with the previous conclusion (Tallman and Zeiger, 1988) and with subsequent corroboration (Talbott and Zeiger, 1993), Poffenroth et al. (1992) attributed the elevation of guard-cell sugar concentration to operation of the PCRP and to starch breakdown. In summary, the work discussed in the preceding 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. We (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, not sources. Although we (Lu et al., 1995) found 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. Presently, 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 substantial sources 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

Plant Material

Vicia faba L. cv Longpod was grown in a growth chamber in Metro-Mix 220 potting medium at a density of two plants per 1-L pot. Illumination was supplied by fluorescent and incandescent lamps (wattage ratio (fluorescent: incandescent) ≈ 6; maximum PAR at plant level ≈ 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Full incandescent illumination began at 0600 h; beginning at 0630 h, fluorescent lamps were turned on at 30-min intervals in three equal lots. The lamps were turned off in steps, beginning at 2100 h, 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) at each watering. The youngest fully expanded bifoliate was used in all 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 comprised four Plexiglas chambers that were connected in series, thus: (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 $GBq \cdot mol^{-1}$). 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 $L \cdot min^{-1}$) through-out the system by a squirrel-cage fan at the inlet of this chamber. (b) the labeling chamber (11 (wide) x 48 (long) x 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 relative humidity. (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 $CO_2 \cdot L^{-1}$ 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 two additional hours, 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 (Fig. 1-6). Because the labeling chamber accepted only three plants, each experiment required two labeling episodes. Three samples from each leaf were taken in order: (a) A 2 x 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) Immediately, 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 (cf. suc wash-out kinetics of Daie, 1985); then, it also was frozen immediately for histochemical analysis ("guard-cell cytoplast"). (c) The sister leaflet was placed in a pressure chamber (Model 100, PMS Instrument Co., USA), which was fitted with a custom seal (M.S. Ewert and W.H. Outlaw Jr., unpublished) 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 in order to show pair-wise comparisons between palisade cells and guard cells of the same leaflet. As shown in the ratio plots, this analysis eliminates potentially confounding differences in CO_2 incorporation among leaflets. A replicate experiment corroborated the displayed results as discussed in the text.

In a previous report (Lu et al., 1995), we referred to the membrane-bound contents of the guard-cell pair as "guard-cell symplast" to connote the guard-cell-pair symplastic domain. Understanding that this terminology is confusing, we use the term "guard-cell cytoplast" here synonymously with our previous term, "guard-cell symplast."

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 (cf. 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

For the displayed experiment, 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 ^{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 Chemical Co.) to remove potentially radioactive organic ions. Then, the neutral supernatant was amended to include 0.1 mM authentic suc and subsequently fractionated by HPLC (CarboPac column, PA1, 4 x 250 mm with 150 mN NaOH mobile phase at a flow rate of 0.8 $mL \cdot min^{-1}$). Authentic-suc elution, which indicated the position of the ^{14}C-suc of the extract, was identified by a pulsed amperometric chromatographic detector.

^{14}C assay was by liquid scintillation counting with special precautions because of the limited amount of ^{14}C present in the displayed results (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, internal standards).

RESULTS AND DISCUSSION

Suc Specific Radioactivities in Palisade Cells and in Petiolar Sap Provide a Basis for Interpreting ^{14}C Abundance in Guard Cells

Earlier, we (Outlaw et al., 1975; Fisher and Outlaw, 1979) reported that suc specific radioactivities in whole leaflet, in palisade parenchyma cells, and in spongy parenchyma cells peak 15 to 20 min after briefly pulse labeling *V. faba* leaflets with $^{14}CO_2$. Those data comprised entire kinetics series of suc specific radioactivities for different suc pools of several individual leaflets; thus, the resulting separate curves were each derived from individual leaflets that served as internal controls for the rate of ^{14}C incorporation (see Outlaw et al., 1975). In the present experiments (including those not shown), the rates of

^{14}C incorporation (soluble fraction) by palisade cells of different leaflets also varied somewhat (equivalent to 86 ± 30 (SE) μmol $CO_2 \cdot mg^{-1}$ chl$\cdot hr^{-1}$, compared with a reference value of 20 to 60 μmol $CO_2 \cdot mg^{-1}$ chl$\cdot hr^{-1}$ for enzymically isolated mesophyll cells of *V. faba* (Outlaw et al., 1976)). This rate of photosynthesis is ~ 14 pmol $CO_2 \cdot cell^{-1} \cdot hr^{-1}$ (conversion factors collected in Outlaw et al., 1985) or 1.3 μmol $CO_2 \cdot cm^{-2}$ leaf area$\cdot hr^{-1}$ (27 mg fresh leaf$\cdot cm^{-2}$ leaf area, unpublished). The suc specific radioactivities of palisade cells of different leaflets harvested at various times during the chase period (the apparent time course in Fig. 1) fit the composite kinetics data that we modeled (Outlaw et al., 1975; Fisher and Outlaw, 1979). Thus, even without the expected requirement to normalize the data to account for differences in ^{14}C incorporation among leaflets, our raw results (Fig. 1) provide a useful quantitative reference for novel data in the following sections. Notwithstanding this apparent fit, we also present the results (Fig. 3-6) as ratios of radioactivities and as ratios of suc specific radioactivities in different pools within the same leaf (cf. Outlaw and Fisher, 1975; Outlaw et al., 1975).

Because the kinetics of suc specific radioactivities in the three mesophyll layers (viz. the palisade parenchyma, the upper spongy parenchyma, and the lower spongy parenchyma) are qualitatively similar (Outlaw and Fisher, 1975; Outlaw et al., 1975), we only assayed palisade cells to provide the basis for comparison. We favored use of palisade cells because they are easiest to select uniformly, they have the highest radioactivity after a pulse of $^{14}CO_2$, and we (Fisher and Outlaw, 1979) have studied them more intensively.

The suc specific radioactivities for whole palisade cells (Fig. 1) derive from two major suc pools, a mobile pool identified as the cytosol but logically including the palisade apoplast also and a relatively non-mobile 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. 7, Outlaw et al., 1975) predicts that the suc specific radioactivity in the palisade cytosol would have peaked at approximately 8 min with a maximum value of 353 $GBq \cdot mol^{-1}$ and would have declined to 167 $GBq \cdot mol^{-1}$ when the whole-cell suc specific radioactivity was maximum, 111 $GBq \cdot mol^{-1}$ (20-min time point, Fig. 1).

Over the course of the experiment, the palisade suc pool size was at steady state (2.6 to 3.3 $pmol \cdot cell^{-1}$). If restricted to the palisade-cell protoplast (46 pL, Willmer and Fricker, 1996), the suc concentration there averaged 65 mM, a composite value for the vacuole plus the cytosol. 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 $GBq \cdot mol^{-1}$ (Fig. 2), occurred at 15 min; i.e., this maximum preceded and was ~ 200x less than that of whole palisade cells (Fig. 1), but lagged behind the predicted peak in the palisade cytosol. Directly comparable data are not available, but these values, along with the kinetics of suc specific radioactivity in leaflet veins (Outlaw and Fisher, 1975; Outlaw et al., 1975), provide new insight into the compartmentation of suc within the leaf. Applied to the present data

(Fig. 1), our model predicts that the suc specific radioactivity in veins should have reached a maximum, 220 GBq·mol^{-1}, at 18 min. As a matter of principle, following a pulse of $^{14}CO_2$, the maximum suc specific radioactivity of the apoplast (an intermediate pool) should be less than that of the mesophyll cytosol (the donating pool) and greater than that of the veins (the receiving pool). Obviously, the petiolar-sap suc specific radioactivity did not fit the principle required of the leaf apoplast. Therefore, an intentionally extreme upper-limit estimate for the maximum suc specific radioactivity of the apoplast in the present experiments was made. This extreme estimate was made from the observed photosynthetic rate, with all ^{14}C incorporated being converted to ^{14}C-suc and deposited instantaneously into the apoplast and by making two assumptions: (a) the leaf apoplast is a homogenous pool (6.6 μL·cm^{-2}, pressure-bomb method, M.S. Ewert, S.Q. Zhang, W.H. Outlaw Jr., unpublished), and (b) the suc concentration in the petiolar sap expressed by the pressure bomb (Fig. 2) is an indicator of the leaf-apoplastic suc concentration. Even this extreme upper-limit estimate of the suc specific radioactivity of a bulk-leaf apoplast (95-154 GBq·mol^{-1}) was lower than the model-predicted maximum suc specific radioactivity for veins (220 GBq·mol^{-1}, above). This incongruity implies that at least one of the two explicit assumptions for the calculation is invalid. Either (a) a lower suc concentration in the apoplast than in the petiolar sap, or (b) a localized apoplastic region between the mesophyll and veins that is not in equilibrium with the bulk-leaf apoplast would be required to bring into accord the model prediction and the upper-limit estimate. We discuss the faults of the two explicit assumptions in the relevant sections, and we show that there is a localized apoplastic region.

The suc concentration of petiolar sap of these photosynthesizing shoot-pruned plants was at steady-state, in the range of 3.1 to 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 by three orders of magnitude (cf. Pomper and Breen (1995), 50 mM, and Fondy and Geiger (1977), 70 μM), but our value is typical (e.g., Lohaus et al., 1995). Importantly, however, the value that we obtain by expressing petiolar sap with the pressure bomb is somewhat lower than the *V. faba* cognate value (9.5 mM) that we calculate from the data of Ntiska and Delrot (1986), who expressed their data on a nEq $C \cdot cm^{-2}$ leaf-area basis. Ntsika and Delrot obtained their apoplastic samples from leaf fragments; one interpretation, therefore, is that the apoplastic concentration of suc near minor veins, the site of phloem loading in *V. faba* (Bouché-Pillon et al., 1994), is *higher* than that near the petiole. The higher value is in accord with the observation that 20 mM suc in the free space region of minor vein of *Beta vulgaris*, also an apoplastic loader, was required to support translocation rates (Sovonick et al., 1974). These two last comparisons are consistent with a *higher* suc concentration in the apoplast of the leaf blade than in the petiolar sap.

In summary, this section establishes (a) a quantitative basis for intraleaflet comparisons of total ^{14}C and ^{14}C-suc among the major source pools and pools in guard cells, which will be shown to be sinks, and (b) the basis for consideration of an apoplastic route for suc from the mesophyll cell cytosol to the guard-cell apoplast.

Incorporation and Import of ^{14}C by Guard Cells of Leaves Pulse Labeled with $^{14}CO_2$.

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 post-labeling period (Fig. 3). (Guard cells dissected from leaflet fragments contain both the cytoplastic and apoplastic pools.) Over the first 20 min of the post-labeling period, guard cells contained relatively little soluble ^{14}C (5.2 ± 1.1 % of that of palisade cells (cell basis)), equivalent to a low overall C incorporation rate (0.013 μmol $CO_2 \cdot cm^{-2}$ leaf area$\cdot hr^{-1}$ ($\approx$ 1 % of that of palisade + spongy mesophyll.)) 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 pmol$\cdot cell^{-1} \cdot hr^{-1}$. Because of the low levels of ^{14}C in the initial samples (net dpm $\approx$ 1.5 dpm per assay), the individual values were imprecise, but, in aggregate, they are in the range of incorporation into organic acids (guard-cell malate accumulation rate: 0.33 pmol$\cdot cell^{-1} \cdot hr^{-1}$, Outlaw and Kennedy, 1978; guard-cell PEP carboxylase activity: 3-4.5 pmol$\cdot cell^{-1} \cdot hr^{-1}$, Tarczynski and Outlaw, 1990; Wang et al., 1994).

The ^{14}C content of guard cells *in situ* increased dramatically between 20 and 40 min ($[^{14}C_{palisade}]/^{14}C_{guard\ cell}] = 23$ at 20 min and 1.3 at 40 min, Fig. 1, and in the replicate experiment, 14 and 2.5, 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. Overall, two conclusions ensue: (a) the kinetics implied that most ^{14}C found in guard cells 40 min post labeling was imported from a different pool, and (b) the relatively low chl

content of guard cells (2.5 pg chl·guard cell^{-1} vs. 158 pg chl·mesophyll cell^{-1}, median of values in Tab. 3.1, Willmer and Fricker, 1996) and the relatively high ^{14}C content of guard cells (in the 40-min sample, 0.36 Bq·guard-cell pair^{-1}, Fig. 3) were inconsistent with a photosynthetic origin of the ^{14}C within guard cells, which would have required a minimum rate of 6800 μmol CO_2·mg^{-1} chl·hr^{-1} (see also Outlaw, 1989, Outlaw et al., 1996).

Import of Mesophyll-derived ^{14}C-suc by Guard Cells of Leaves Pulse Labeled with $^{14}CO_2$.

Figure 4 shows the apparent time course of guard-cell suc specific radioactivity. These 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 (following section) 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 radioactivy was only 0.07-fold that of palisade cells at 20 min, but the ratio increased to 5.4 at 40 min).

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) and did not increase or decrease over the course of the experiment. The steady state in suc concentration at this time in the photoperiod was in confirmation of our previous results (Lu et al., 1995) for leaves grown and harvested under these conditions, but was 2-fold more than the

concentration of suc in guard cells of leaves under low-CO_2 conditions (Outlaw and Manchester, 1979). This difference is consistent with a photosynthetic origin of suc, but not, as noted, in guard cells.

Distinction of the Mesophyll-derived ^{14}C-suc Pools of the Guard-cell Cytoplast and Guard-cell Apoplast of Leaves Pulse Labeled with $^{14}CO_2$.

The apparent time course for guard-cell cytoplastic suc specific radioactivity was determined by assaying extracts of cells dissected from rinsed epidermal peels harvested during the post-labeling period (Fig. 5A). As discussed in the previous section for the whole-cell data, 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 cytoplastic suc-specific radioactivities during the first 15 min of the post-labeling period (Fig. 5A, 5B) to have biological significance. For the 20-min post-labeling sample, in which the palisade-cell suc specific radioactivity was maximum, guard-cell cytoplastic suc specific radioactivity remained low, 0.3-fold that of palisade cells (Fig. 5A; 0.24-fold in the replicate experiment). At this time, the guard-cell cytoplast contained approximately 7 % as much ^{14}C-suc as did palisade cells. The guard-cell cytoplastic suc specific radioactivity of the 40-min post-labeling sample was dramatically higher than that at 15- or 20-min post labeling (Fig. 5A), mimicking the pattern for the total soluble ^{14}C. More significantly, and a major element of this report, the ratio (guard-cell cytoplastic suc specific radioactivity)/(palisade-cell suc specific radioactivity) increased to 1.2 at 40-min post-labeling (Fig 5A; 1.4 in the replicate experiment), and to 3.6 at 60-min post-labeling.

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). The kinetics did not permit a calculation of the rate at which ^{14}C-suc was imported into guard cells because assumptions concerning the instantaneous relevant apoplastic suc specific radioactivities are problematic, as discussed earlier, and because the time resolution after 20 min was poor. It was possible, however, to calculate a lower limit (12-21 fmol·min^{-1}·stoma^{-1}; by two methods, net influx and exponential decline). As 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), and is about equivalent to suc uptake by isolated guard-cell protoplasts of *Commelina* with 0.5 mM suc external (Reddy and Rama Das, 1986). On 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) and is in the same range as suc uptake rates by *V. faba* leaf fragments (Delrot (1981), whose data we converted from a leaf-surface-area basis). As 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 relevance of competition between K^+ uptake and suc uptake by guard cells. As perspective in the context of carbon metabolism associated with stomatal movements, the lower limit for guard-cell suc uptake was sufficient to replenish in as little as 20 min the starch degraded during stomatal opening (see Outlaw and Manchester, 1979).

As perspective in the context of guard-cell heterotrophy, the lower limit calculated for guard-cell suc uptake was more than 15-fold the amount of carbohydrate required to support respiration (median of literature values collected in Tab. II, Gautier et al., 1991)

The suc content of the guard-cell cytoplast (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 cytoplastic suc concentration under the present conditions did increase from dawn to mid-day, but the osmolarity change was relatively minor in the context of stomatal movements unless suc accumulation is restricted to a small region of the cytoplast.

The apparent time course for guard-cell apoplastic suc-specific radioactivity (Fig. 6A, 6B) was constructed from data for the whole-cell (Fig. 4) and for the guard-cell cytoplast (Fig. 5A). The guard-cell apoplastic suc-specific radioactivity increased remarkably during the period 20- to 40-min post labeling to 204 GBq·mol^{-1} (Fig. 6A), which was higher than that of guard cells (154 GBq·mol^{-1}, 40 min, Fig. 4) and 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 palisade cells. Following the principle outlined above, the suc specific radioactivity of the guard-cell apoplast was lower than that predicted for the mesophyll cytosol (353 GBq·mol^{-1}), the putative donor pool. In confirmation that the entire volume of the leaf apoplast did not serve as a homogenous intermediate between the mesophyll cytosol and the guard cell, the guard-cell-apoplastic suc-specific radioactivity

was *higher* than the extreme upper-limit estimate for the apoplastic suc-specific radioactivity (95-154 GBq·mol^{-1}, see "Suc specific radioactivities in the palisade . . . ."). Altogether, our data indicated that ^{14}C-suc excreted from the mobile suc pool in the mesophyll moved in the apoplastic solution to the terminal point in the evaporative pathway, the guard-cell wall. Thus, following a brief pulse of $^{14}CO_2$, the apoplastic suc specific radioactivity should be highest around the mesophyll cells and then in the apoplast between the mesophyll and guard cells. Lower specific radioactivities should obtain in the apoplast most distal to mesophyll, i.e., in petiolar sap, which is in accord with our observations (0.54 GBq·mol^{-1}). As a first-order estimate at the time resolution of these experiments and the model, nominally 30 min separated the peaks of suc specific radioactivities in the mobile-palisade suc pool and the guard-cell suc apoplastic pool. During this time, transpirational water loss was estimated to be 3.1 μL·cm^{-2} (9600 stomata·cm^{-2}, 10 pL·min^{-1}·stoma^{-1}, 32 min). Use of this value as the apoplastic volume into which the ^{14}C-suc was diluted upon excretion from the mesophyll indicates a maximum suc specific radioactivity in the localized apoplast continuous with the guard-cell wall of 202- to 327 GBq·mol^{-1}. Thus, consistent with our paradigm, this calculated suc specific radioactivity was less than the maximum value of the mobile pool of the mesophyll (353 GBq·mol^{-1}) and is equal to or more than the maximum value of the guard-cell wall (204 GBq·mol^{-1}). In other work, we have shown that ABA fed by petiolar injection (S.Q. Zhang, W.H. Outlaw Jr., K. Aghoram, unpublished) or mannitol fed via the petiole (M.S. Ewert, S.Q. Zhang, W.H. Outlaw Jr., unpublished) was also concentrated in the guard-cell apoplast, which is easiest explained by the absence of

significant interfering "sumps" (see Canny, 1995) in *V. faba* leaflets. If, as suggested, apoplastic discontinuities are not apparent, the directed movement of suc toward the guard-cell wall, and not into the apoplast at large, could be explained by differences in suc diffusivity in different cell walls, by apical bulk flow in the major veins and perhaps along cell wall surfaces, by peristomatal transpiration, and by the chromatography effect (see "Conclusions").

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 an suc efflux rate of 0.05 pmol $\cdot stoma^{-1} \cdot min^{-1}$. (Important to our interpretation (see "Conclusions"), this rapid loss of sucrose from the guard-cell apoplast indicates that the pool size would decrease rapidly in the absence of a high input rate.) The guard-cell apoplastic suc pool was at steady-state (Fig. 6A) and, thus, the influx rate was equal to the efflux rate. It is important to recognize, however, that the guard-cell apoplast is not a simple pool with a single solution input and a single solution output. Instead, we explain suc gain in solution, but solvent loss from this terminal point by evaporation. Under experimental conditions, the movement of apoplastic water to guard cells was 10 $pL \cdot min^{-1} \cdot stoma^{-1}$ (calculated from a typical conductance value using the prevailing driving force for water loss). In other words, the solvent turnover rate in the guard-cell apoplast was more than 24-fold that of the solute.

The suc content of the guard-cell apoplast (Fig. 6A, 6B) was 0.9 to 1.3 pmol$\cdot$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, W.H. Outlaw Jr.; reconstruction of serial sections, unpublished). Taking 0.3 mL ·cm^{-3} as a typical value for the water-free-space: cell-wall volume ratio (Dainty and Hope, 1959; Senden et al., 1994; Fleischer and Ehwald, 1995), we estimated the water-free space volume of *V. faba* guard-cell walls to be 2.5 pL·stoma^{-1}. Thus, (a) use of the estimated water-free space volume, (b) conversion of suc content (mid-day, present experiments + those of Lu et al., 1995) to a molality basis (0.6 mL·gm^{-1}, solute-volume displacement), and (c) correction for the variable osmotic coefficient of suc (Michel, 1972), we calculated that Ψ_s(suc) in the guard-cell wall changed by -1.4 MPa from dawn to mid-day. Using the average of the $\Delta\Psi_s/\Delta\mu$m aperture relationships of Fischer (1972) and Raschke (1979) for *V. faba*, we conclude that the presence of guard-cell-wall suc derived from the apoplast would depress stomatal-aperture size by 4 μm. (Because of the several conversions required in this calculation, we consider it a reasonable estimate, and not a precise value.) In conclusion, mesophyll-derived suc is important quantitatively as an external osmotic regulator of stomatal-aperture size in the intact leaf of *V. faba*; it may have other effects also, e.g., on gene expression (Thomas and Rodriguez, 1994). Finally, we note that other apoplast-derived metabolites, notably hexoses from the acid-catalyzed hydrolysis of suc, malate (see Hedrich and Marten, 1993), and ABA (S.Q. Zhang, W.H. Outlaw Jr., unpublished), may accumulate in the guard-cell wall as a result of transpiration and have osmotic or signal-transduction effects on stomatal-aperture size.

CONCLUSIONS

Our summary interpretation is presented in Figure 7. 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 (see van Bel, 1993). Further movement of fluid, through the porous walls of mesophyll cells, is also by diffusion (Tyree and Yianoulis, 1980), but bulk flow along the surfaces of hydrated walls cannot be discounted. Most evaporation of the transpiration stream occurs in or 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). Regardless, however, of the sites of evaporation, 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 (references in van Bel, 1993)) and efficiency of transport from the leaf (demonstrated by blockage of translocation (Lohaus et al., 1995, and references therein)). Thus, our first hypothesis is that high apoplastic suc concentration is a signal that causes a diminution of stomatal-aperture size. This effect may not be immediate because of the nominal 30-min lag time required for suc movement from the mesophyll cytosol to the guard-cell wall, and we realize that other

physiological controls, such as the partitioning ratio, would also interact with the proposed signaling. The second factor, transpiration rate, has been elegantly shown (Mott and Parkhurst, 1991) to be a means by which plants respond to humidity (see additionally 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, but this hypothesis could be modified if subsequent investigations reveal a lack of concordance between the kinetics of stomatal closure in response to an elevated transpiration rate and suc accumulation in the guard-cell apoplast. Obviously, other factors such as intraleaf gradients in water potential (e.g., Shackel and Brinckmann, 1985) not shown in Figure 7 constitute other mechanisms of stomatal-aperture size regulation in intact leaves. The actual aperture size, thus, results from an integration of all these factors.

Even a qualitative interpretation of an attenuation of stomatal-aperture size in an ecological context is hazardous. In addition, the quantitative relationship between leaf conductance, of which stomatal conductance is one term, and stomatal-aperture size depends on other independent variables such as wind and leaf morphology. With those reservations, we note that 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 (e.g. Lakso, 1994), 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, say, $g = 0.3$ 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 but only minor reduction in photosynthetic rate.

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FIGURE LEGENDS

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 *Vicia 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 SEM 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 time-points, respectively, were also used for dissection of other displayed cell data (Fig. 3-6).

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

Figure 3. The water-soluble ^{14}C content of guard cells (Δ) 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 (Δ). On the basis of the ^{14}C in the 5-, 10-, and 15-min time points and the specific radioactivity of the $^{14}CO_2$ used, the ^{14}C incorporation rate estimate was 0.7 pmol·guard cell^{-1}·hr^{-1}. This rate estimate is imprecise because of the percent counting error in determining the low dpm in the initial guard-cell samples. The main value of this estimate is its use as a benchmark for comparison with the ^{14}C incorporation rate of palisade cells (14 pmol·cell^{-1}·hr^{-1} ≈ 86 μmol mg^{-1} chl·hr^{-1}). The error bar is the largest SEM for replicate radioactive assays (n = 6). For other details, see Figure 1. Each time point for all displayed data for guard cells (Fig. 3-6) were 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 cytoplastic- and apoplastic-suc compartments. The error associated with suc specific radioactivity is the largest SEM for replicate radioactive assays (n = 7). For other details, see Figures 1 and 3.

Figure 5A. The suc specific radioactivity (□) and suc content (■) of the guard cell-cytoplast 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 SEM for replicate radioactive assays (n = 7). For other details, see Figures 1 and 3.

Figure 5B. The ratio of the guard-cell-cytoplast suc specific radioactivity (Fig. 5A) 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 (o) is the ratio using the maximum palisade-cell suc specific radioactivity (20 min, Fig. 1). For other details, see Figures 1 and 3.

Figure 6A. 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 cytoplastic data (Fig. 5A). For other details, see Figures 1 and 3.

Figure 6B. 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 (o) is the ratio using the maximum palisade-cell suc specific radioactivity (20 min, Fig. 1). For other details, see Figures 1 and 3.

Figure 7. Mesophyll-derived suc accumulates in the guard-cell walls of open stomata because of the chromatography effect (see Tyree and Yianoulis, 1980; solutes accumulate at the distal point in an evaporative pathway) and peristomatal evaporation (see Maier-Maercker, 1983; evaporation from or near guard cells). This model, with the darker regions representing pools rich in recently synthesized sucrose, indicates that high

transpiration rates (see 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.

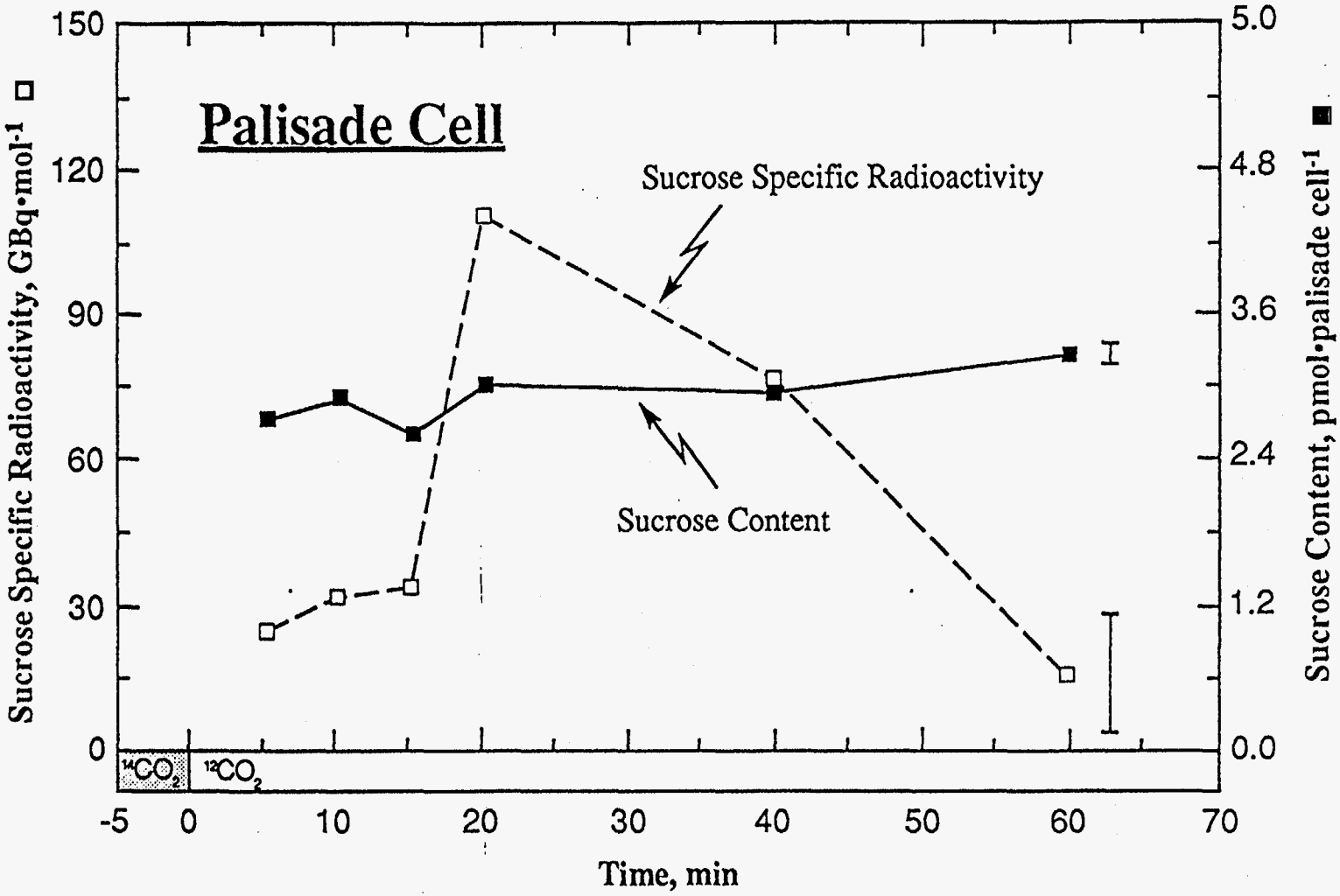
Palisade Cell
Sucrose Specific Radioactivity
Sucrose Content
Sucrose Specific Radioactivity, GBq•mol-1
Sucrose Content, pmol•palisade cell-1
Time, min
14CO2
12CO2
150
120
90
60
30
0
5.0
4.8
3.6
2.4
1.2
0.0
-5
0
10
20
30
40
50
60
70

Fig 1
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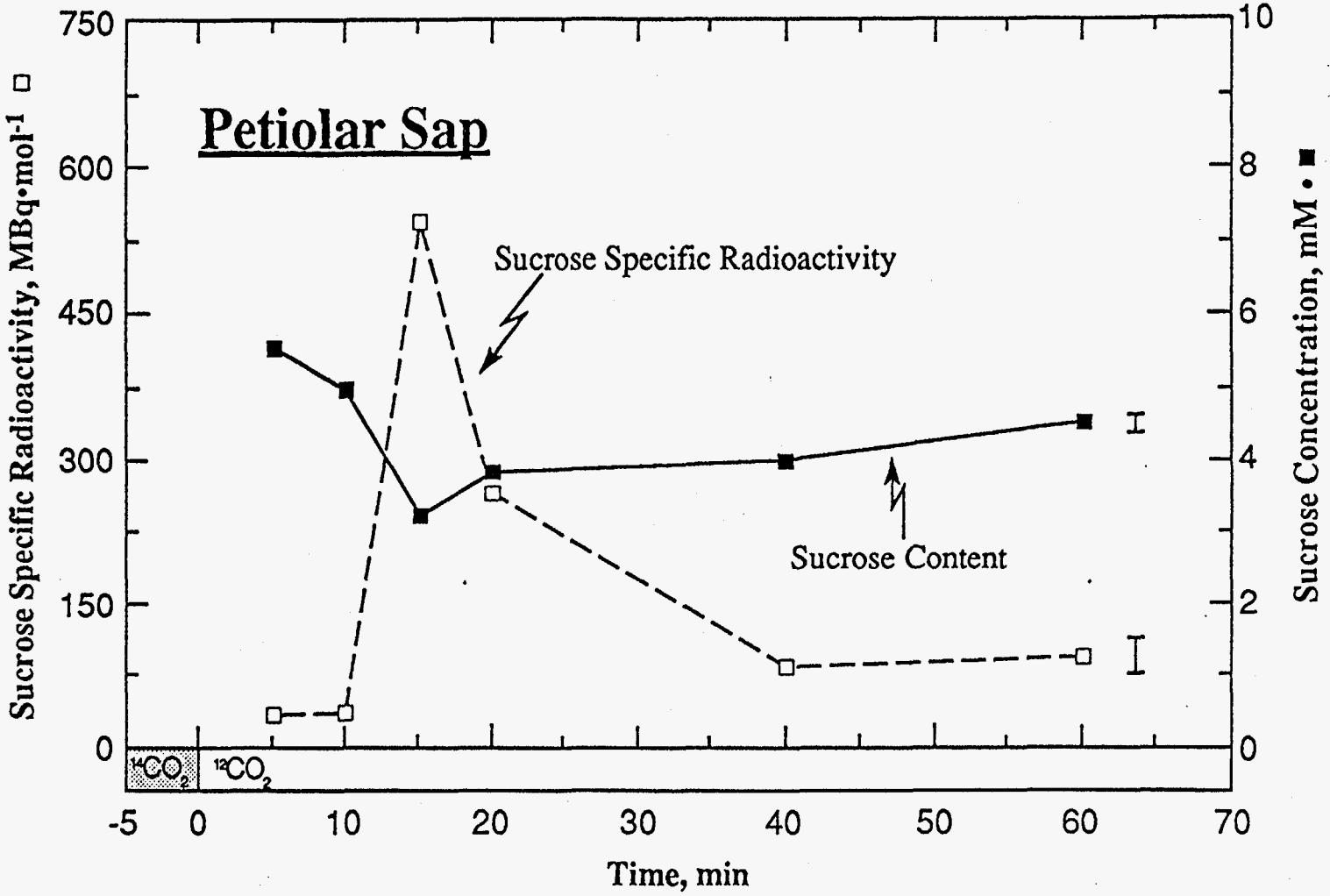
Petiolar Sap
Sucrose Specific Radioactivity
Sucrose Content
Sucrose Specific Radioactivity, MBq•mol-1 □
Sucrose Concentration, mM • ■
Time, min
14CO2
12CO2
750
600
450
300
150
0
10
8
6
4
2
0
-5
0
10
20
30
40
50
60
70

Fig 2
Lu et al.

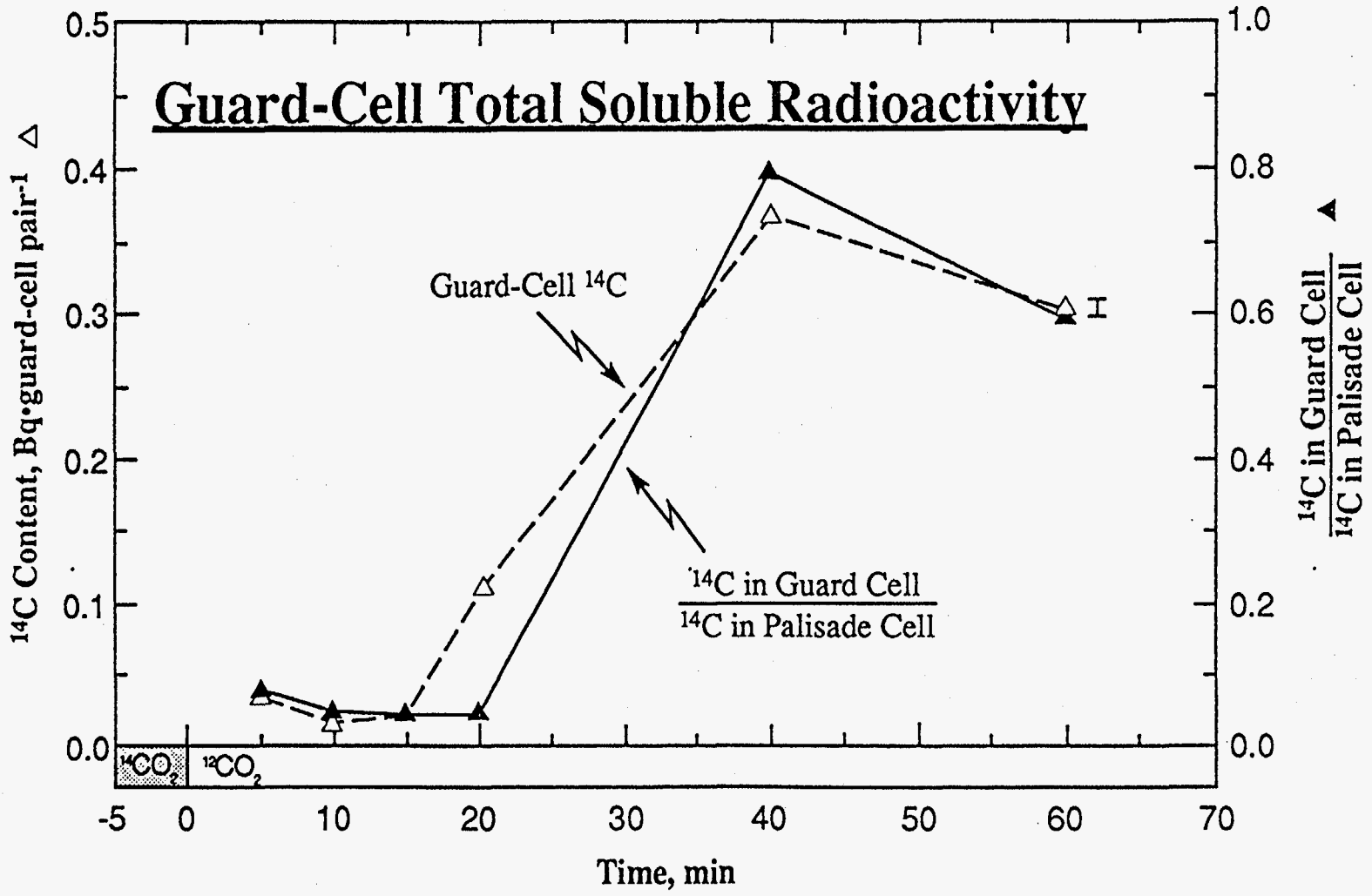
Guard-Cell Total Soluble Radioactivity
^{14}C Content, Bq•guard-cell pair^{-1} △
$\frac{^{14}C \text{ in Guard Cell}}{^{14}C \text{ in Palisade Cell}}$ ▲
Guard-Cell ^{14}C
$\frac{^{14}C \text{ in Guard Cell}}{^{14}C \text{ in Palisade Cell}}$
$^{14}CO_2$
$^{12}CO_2$
0.0
0.1
0.2
0.3
0.4
0.5
0.0
0.2
0.4
0.6
0.8
1.0
-5
0
10
20
30
40
50
60
70
Time, min

Fig 3
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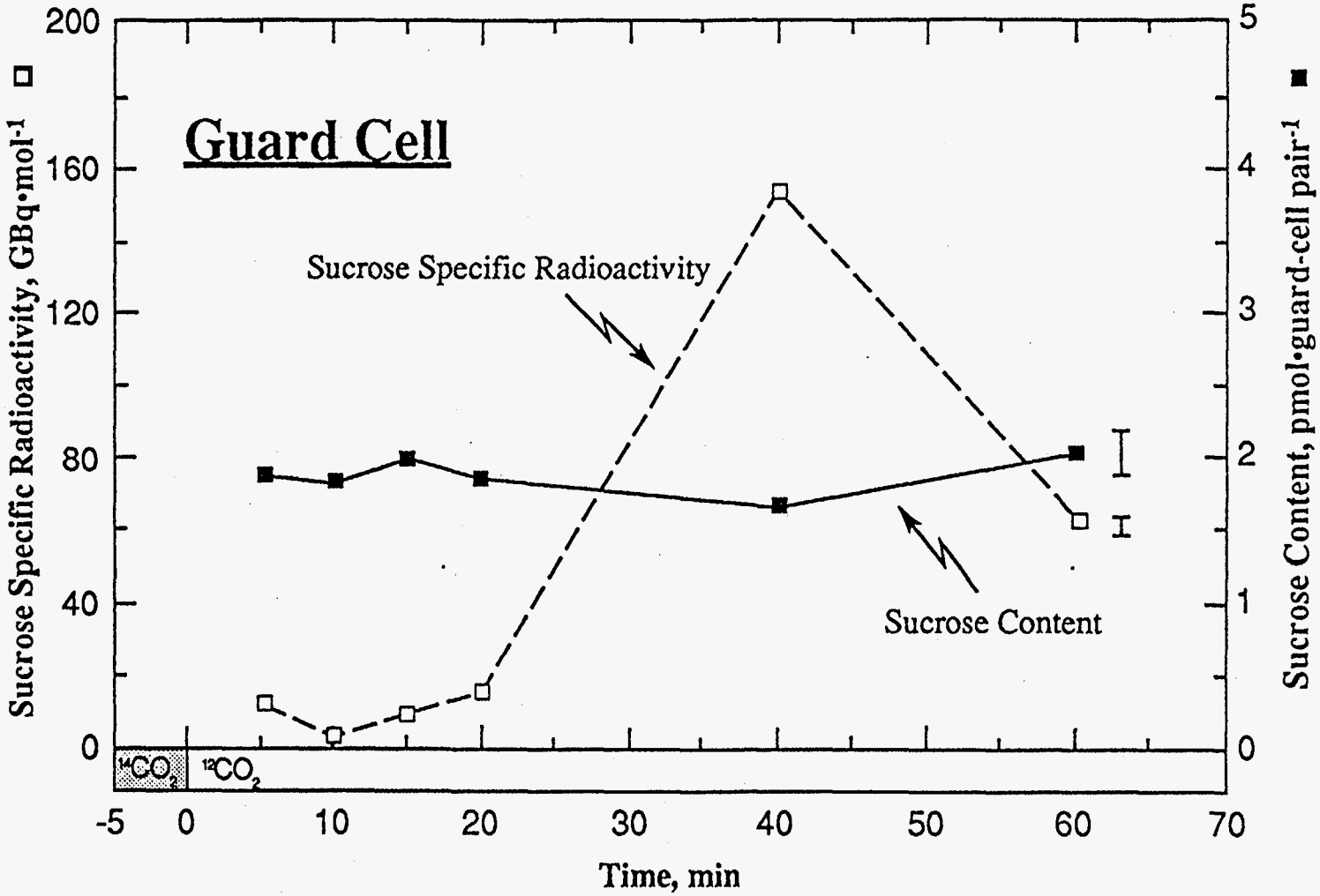
Guard Cell
Sucrose Specific Radioactivity
Sucrose Content
Sucrose Specific Radioactivity, GBq•mol-1
Sucrose Content, pmol•guard-cell pair-1
Time, min
14CO2
12CO2
200
160
120
80
40
0
5
4
3
2
1
0
-5
0
10
20
30
40
50
60
70

Fig 4
Lu etal

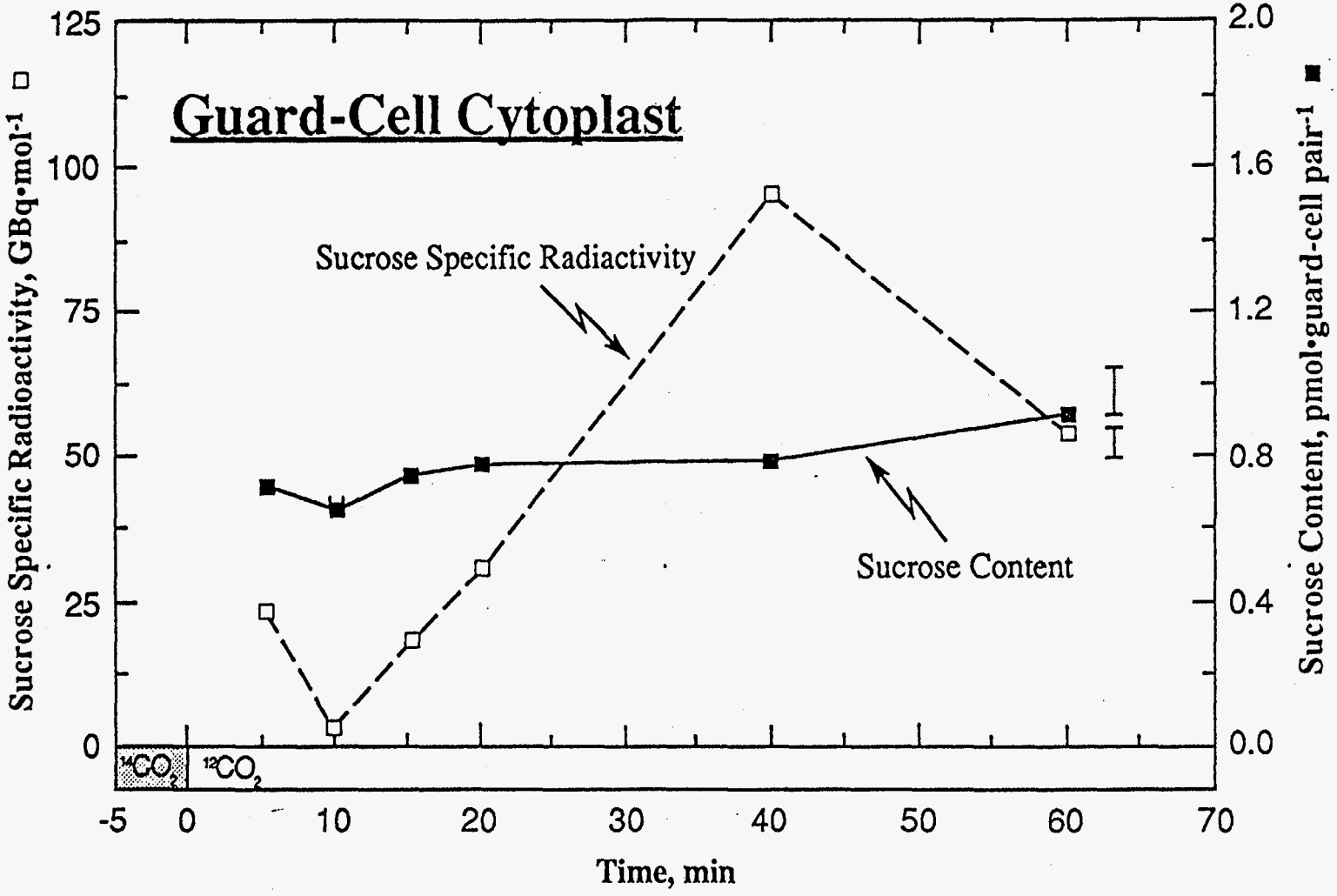

Fig5A
Lu et al.

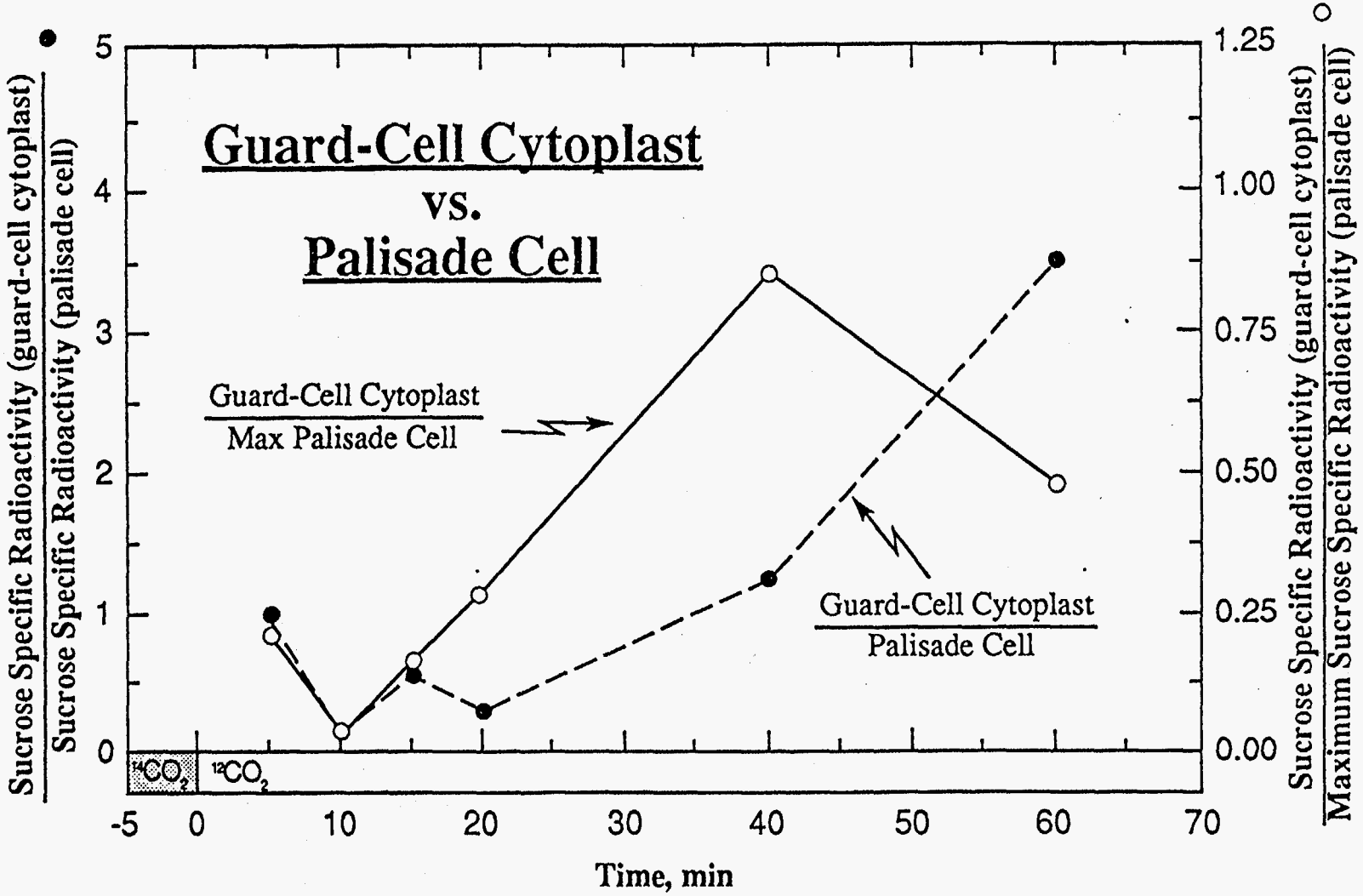

Fig 5B
Lu et al.

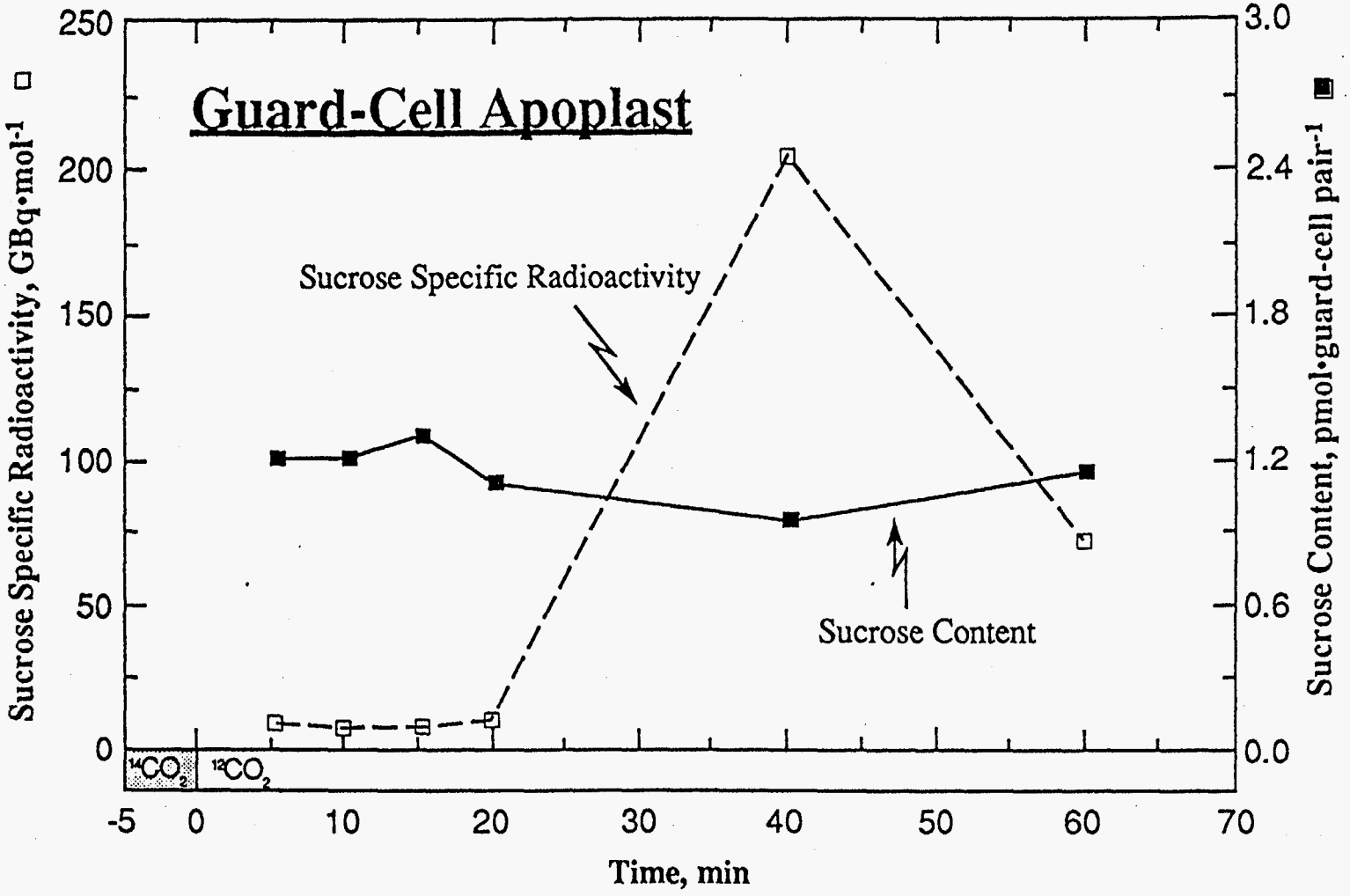

Fig 6A
Lu et al.

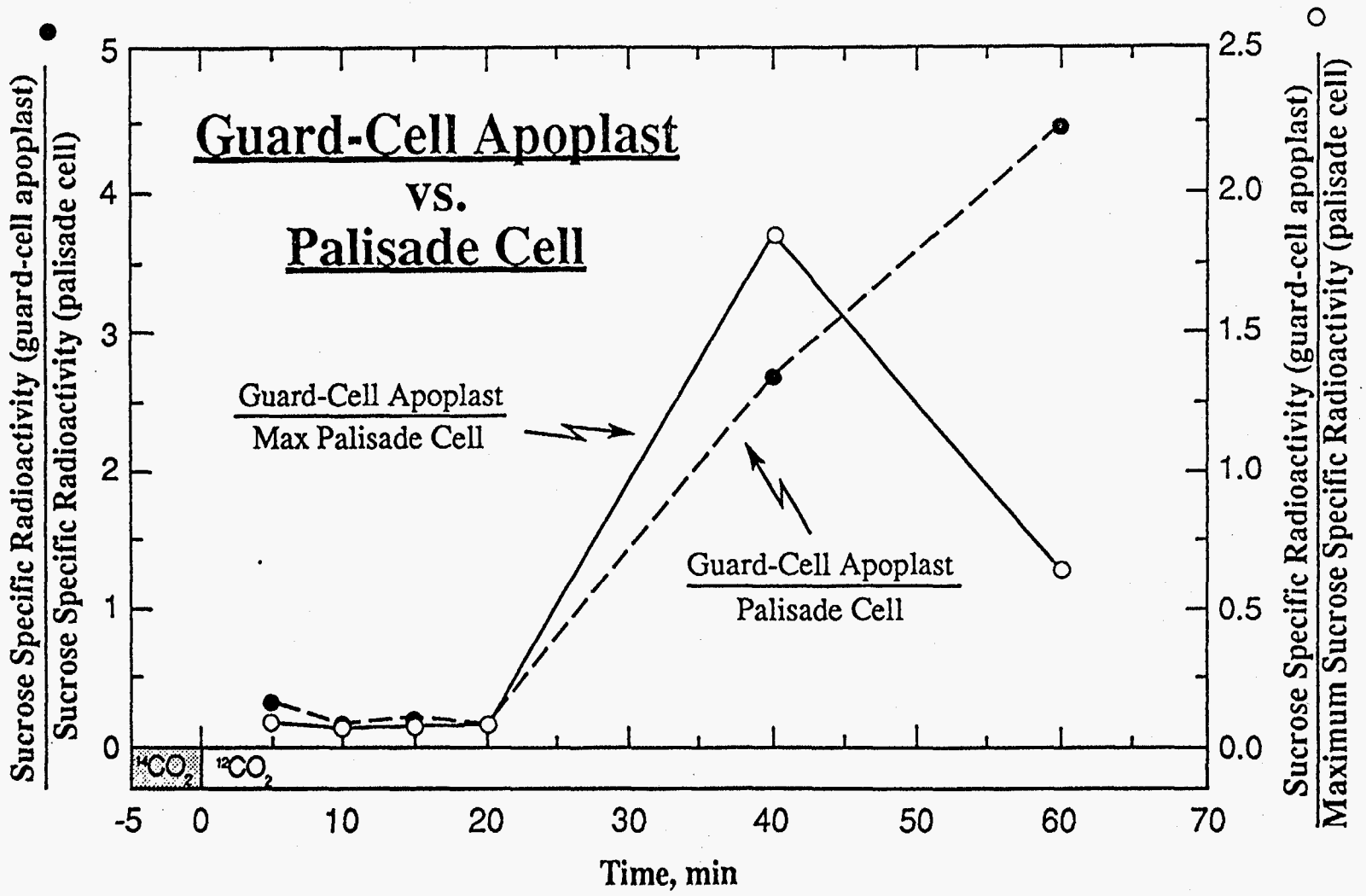

Fig 6B
Liu et al

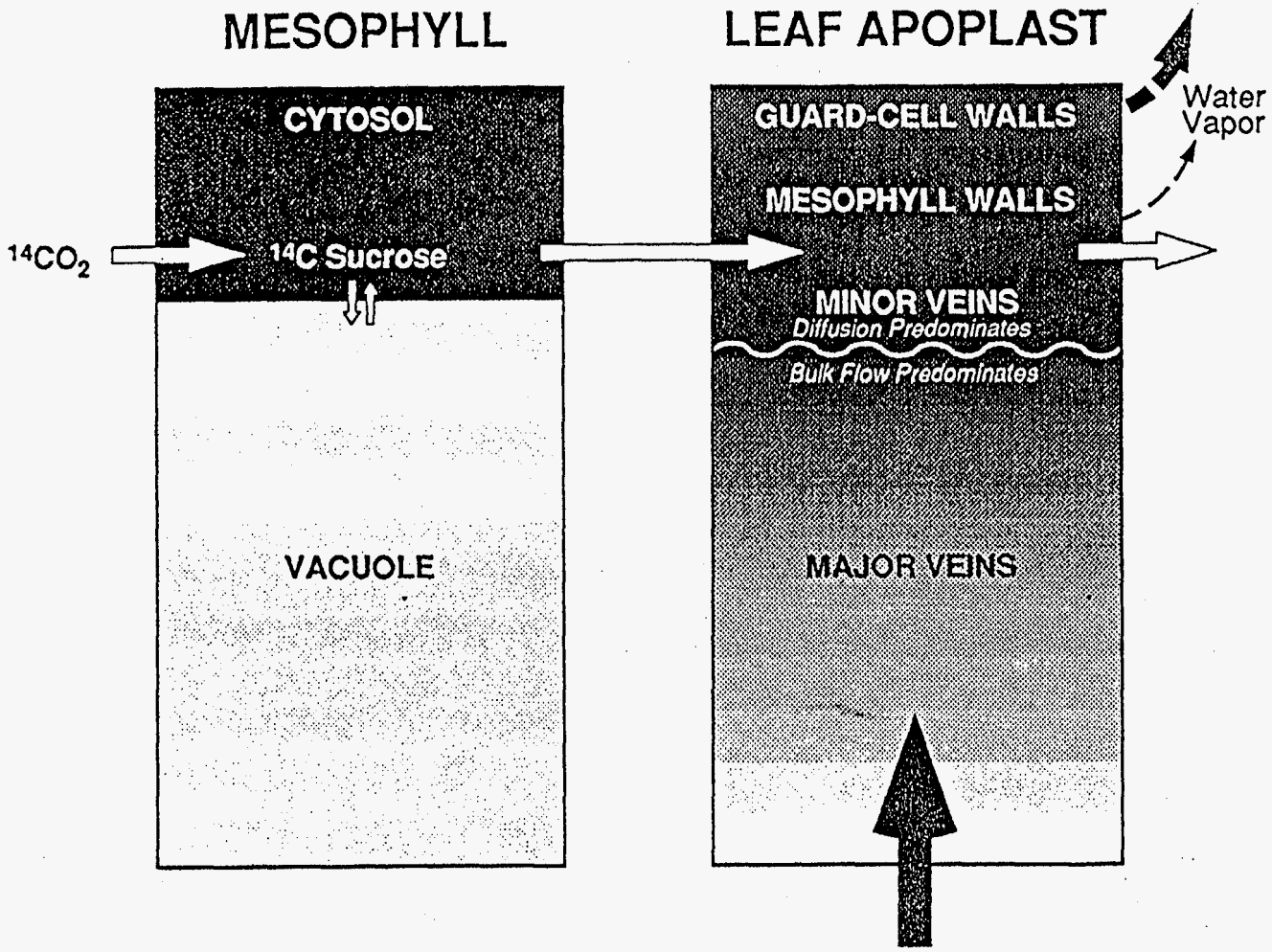
MESOPHYLL
LEAF APOPLAST
CYTOSOL
GUARD-CELL WALLS
Water Vapor
MESOPHYLL WALLS
$^{14}CO_2$
^{14}C Sucrose
MINOR VEINS
Diffusion Predominates
Bulk Flow Predominates
VACUOLE
MAJOR VEINS

Fig 7
Lu et al.