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Monitoring Phloem Unloading and Post-Phloem Transport by Microperfusion of Attached Wheat Grains[1]

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Phloem unloading and post-phloem transport in developing wheat (*Triticum aestivum* L.) grains were investigated by perfusing the endosperm cavities of attached grains. Relative unloading ratio (*RUR*) and the rate of sucrose release into the endosperm cavity (*SRR*) were calculated, respectively, from ^{14}C import and from sucrose washout from the cavity. *RUR* and *SRR* continued at or near in vivo rates over a wide range of cavity sap osmolality (90 to approximately 500 milliosmolal) and sucrose concentration (14–430 mM) and for long times (29 h). These are much greater ranges than have been observed for the endosperm cavity in vivo (230–300 milliosmolal, and 40–120 mM, respectively), indicating that neither the cavity sap osmolality nor sucrose concentration are controlling factors for the rate of assimilate import into the cavity. The maintenance of in vivo transport rates over a wide range of conditions strongly implicates the role of transport processes within the maternal tissues of the wheat grain, rather than activities of the embryo or endosperm, in determining the rate of assimilate import into the grain. *RUR* was decreased by high concentrations of sucrose and sorbitol, but not of mannitol. By plasmolyzing some chalazal cells, sorbitol appeared to block symplastic transport across the crease tissues, but neither sucrose nor mannitol caused plasmolysis in maternal tissues of attached grains. The inhibition of *RUR* by KCN and carbonyl cyanide *m*-chlorophenyl (CCCP) and the continued import of sucrose into grains against its concentration gradient suggest that solute movement into the endosperm cavity might occur by active membrane transport. However, the evidence is weak, since KCN and CCCP appeared to act primarily on some aspect of symplastic (i.e. nonmembrane) transport. Also, sucrose could move from the endosperm cavity into the maternal tissues (i.e. opposite to the normal direction of sucrose movement), suggesting that transmembrane movement in the nucellus may be a reversible process. Pressure-driven flow into the grain could account for movement against a concentration gradient.

Although nutrient import by sinks is a major determinant of assimilate partitioning (Gifford and Evans, 1981), the processes involved and their possible regulation are poorly understood. Experimental investigation is complicated by the simultaneous operation of several transport processes (Oparka, 1990). In seeds, these include movement out of the sieve element-companion cell complex (phloem unloading in the strict sense); post-phloem intercellular transport through several distinct tissues; transmembrane movement into the apoplast; uptake by embryonic tissues; and, finally, utilization of assimilates for growth and/or storage. It is difficult to study these processes separately. To analyze the mechanisms and control of the assimilate import into a sink, transport rates and their relationship to potential driving forces (e.g. gradients in concentration, pressure, etc.) should be measured and the site(s) and pathway(s) of the component transport steps should be established.

These goals have been partially achieved by the surgical removal of embryonic tissues from the seed coat (Patrick, 1983; Thorne and Rainbird, 1983; Wolswinkel and Ammerlaan, 1983) or from maize pedicels (Porter et al., 1985) to intercept solutes being released from maternal tissues into the apoplast. Results from this approach have implicated transport within maternal tissues as a controlling factor for the rate of seed growth (Wolswinkel, 1992). Assimilate import into the ovules of legumes, the most extensively studied species, increases with increasing apoplastic osmotic concentration. Since these treatments presumably alter phloem turgor, apoplast osmotic concentration has been suggested as a possible control factor for solute import into developing seeds (Wolswinkel, 1985, 1990; Patrick, 1990). Also, several workers have shown that solute release from legume seed coats is metabolically dependent (Patrick, 1983; Wolswinkel and Ammerlaan, 1983; Gifford and Thorne, 1986), suggesting the possible involvement of an active transport step(s). Corn, however, contrasts with other species in both respects. Solute release from the corn pedicel is not affected by metabolic inhibitors or PCMBS (Porter et al., 1985) and is decreased by osmotic treatments (Porter et al., 1987).

Despite such substantial advances in understanding assimilate import in the "empty seed coat" and "empty pedicel" systems, they nevertheless have some disadvantages. Pressure, solute concentrations, osmotic potential, pH, and other transport-related parameters in the apoplast or in sieve tubes cannot be measured directly, and estimates by different research groups vary considerably (Hsu et al., 1984; Gifford and Thorne, 1985). At least in legumes, the site of solute release from maternal tissues is not clear; nor is the site of

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; mOsm, milliosmolal; PCMBS, *p*-chloromercuribenzene sulfonate; *RUR*, relative unloading ratio; *SRR*, sucrose release rate.

action of even nonpermeant inhibitors such as PCMBS (Minchin and Thorpe, 1990). Maternal pool sizes and rates of phloem transport often (Gifford and Thorne, 1986; Ellis and Spanswick, 1987; Minchin and Thorpe, 1989; Ellis et al., 1992), but not always (Wolswinkel, 1992), decline with time. These uncertainties complicate the interpretation of treatment effects, especially in distinguishing between phloem unloading in the strict sense (i.e. movement from the sieve tube-companion cell complex) and solute release from the maternal tissues.

Compared with other systems used to date, developing wheat (*Triticum aestivum* L.) grains offer some basic advantages for investigating the mechanism and control of assimilate import: (a) transport-related parameters can be measured in the sieve tubes and in the apoplast (endosperm cavity) between the maternal and embryonic tissues (Fisher and Gifford, 1986); (b) the rate of import is constant and stable over a period of several weeks; (c) short-term measurements of assimilate transport to the ear can be made (Fisher, 1990); (d) the presence of an apoplastic barrier in the chalaza (Zee and O'Brien, 1970b) between the grain phloem and the endosperm cavity greatly simplifies questions concerning apoplastic solute movement of both assimilates and applied nonpermeant solutes; (e) the site of solute release (i.e. the nucellus) from the maternal tissue into the apoplast is apparent; and, finally, (f) a substantial number of replicate grain samples can be obtained from a single ear (Fisher and Gifford, 1986).

Earlier, we investigated the dynamics of Suc pools and their location in maternal tissues of the wheat grain through which assimilates move to the endosperm cavity (Fisher and Wang, 1993). Virtually the entire Suc content of the crease tissues was involved in post-phloem transport, behaving basically as a single, well-mixed transport compartment between the phloem and the endosperm cavity, with a turnover time of about 1.3 h. Since experimental treatments that alter transport rates may differ in their effects on Suc movement into and out of this pool, changes in the pool size should provide a useful indicator of the transport step(s) most affected by the treatments.

The purpose of the research presented here was to develop an experimental system to measure the rate of assimilate import into individual wheat grains and to assess the effects of apoplast osmotic potential, Suc concentration, and metabolic inhibitors on phloem unloading and post-phloem transport. We approached this by perfusing the endosperm cavity of attached wheat grains with various solutions to monitor the processes of phloem unloading and post-phloem transport. The structure of the grain allows perfusion tubes to be inserted into the cavity of attached grains virtually without damage. A preliminary report appears in Patrick et al. (1991).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wheat plants (*Triticum aestivum* L. cv SUN 9E) were grown in a growth chamber. Growth conditions were as described in previous publications (Fisher, 1990; Fisher and Wang, 1993). Briefly, plants were grown on a 16-h photoperiod at a PPFD of 450 μmol m^{-2} s^{-1} and a day/night temperature regime of 22/16°C. Plants were irrigated with water at 16-h intervals and with a complete nutrient solution once a week. Ears were tagged at anthesis and used for experiments 17 to 22 d later.

Structural Studies

The tissues in the crease region were examined for possible plasmolysis in several experiments in which the endosperm cavity was perfused with concentrated sugar solutions. Crease tissues from the perfused grains were quick-frozen in isopentane/methylcyclohexane cooled with liquid nitrogen, freeze-substituted in methanol (Fisher and Housley, 1972), and embedded in L.R. White resin (Polysciences, Warrington, PA) according to the supplier's instructions. Samples were frozen after 2 h (attached grains perfused with 1490 mOsm Suc) and 5 h (all other experiments). Sections 2 μm in thickness were stained with toludine blue O.

The distal end of the grain was examined to clarify the anatomical structures through which the perfusion tubes were being inserted. The distal one-fourth of several grains were quick-frozen and embedded as described above. Sections 1 μm in thickness were stained for carbohydrates by the periodic acid-Schiff reaction or for proteins by Coomassie blue.

Perfusion Experiments

A perfusion pump was constructed to allow the simultaneous perfusion of four grains on a wheat ear, at rates of several μL h^{-1}. It consists of four digitally controlled stepping motors, each driving a screw-type 0.5-mL syringe. The syringes were water-jacketed to prevent thermally generated fluctuations in the perfusion rate. Flow rate was calibrated with *p*-nitrophenol dye (Lowry and Passonneau, 1972) and was accurate to within ± 3% (SD) over the range of 0.4 to 25 μL h^{-1}. Standard perfusion rate was 6 μL h^{-1} $grain^{-1}$. This compares with the endosperm cavity volume of about 2 to 6 μL. The perfusion solution contained 14 mM Mes-KOH buffer, 2 mM K-PO_4 buffer, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, plus various concentrations of mannitol, sorbitol, or Suc to adjust the osmolality. The pH was 6.5. The osmotic concentration of "standard perfusion medium" was adjusted to 300 mOsm, close to that in the endosperm cavity of attached grains (Fisher and Gifford, 1986).

The experimental plant was laid horizontally under a water-filtered metal halide lamp providing a PPFD of 400 to 450 μmol m^{-2} s^{-1}. Four A or B grains (i.e. one of the two lowest grains) on spikelets in the central third of the ear were chosen for each perfusion experiment. The distal ends of the glumes were cut off with a razor and the loose outer pericarp covering the end of the grain was pulled off with forceps. This exposed a transparent layer, about 2 to 3 mm in diameter, covering the end of the endosperm cavity. (Diagrams of cross- and longitudinal sections of a wheat grain are shown in Figure 1.) As shown by anatomical investigations (not reported), this covering consists of only a single layer of living cells (the testa), plus two to three layers of nonliving cells.

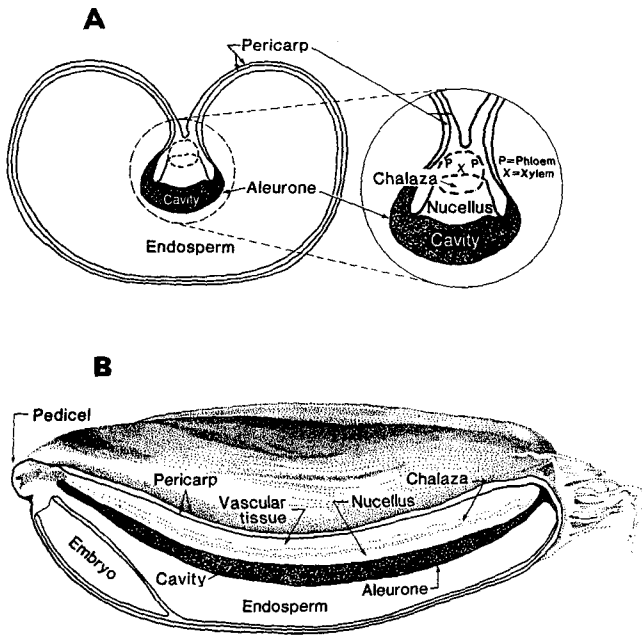

Figure 1. Diagrams of cross- and longitudinal sections of a wheat grain. A, Location of tissues in a cross-section. B, Location of tissues in a longitudinal (sagittal) section.

Gelatinous material, apparently of carbohydrate, filled the distal-most 2 mm of the endosperm cavity, considerably simplifying the problem of obtaining a seal around perfusion tubes. Inlet and outlet tubes (polyimide-coated silica tubing, 150 μm o.d. and 100 μm i.d.; Polymicro Technologies, Phoenix, AZ) were pushed through the covering layer into the endosperm cavity with an inserting depth of 4 to 5 mm and 2 mm, respectively. The tubes were sealed in place with a small drop of cyanoacrylate adhesive. Setup was usually finished within 30 min. All manipulations were done under a dissecting microscope.

In most experiments, the distal third of the flag leaf was allowed to assimilate 50 μCi of $^{14}CO_2$ (generated from [^{14}C]$NaHCO_3$ with a specific activity of 56 mCi mmol^{-1}) for 10 min in a sealed chamber 20 to 30 min after perfusion was initiated. In earlier experiments, however, the flag leaf blade was labeled for technical reasons before inserting the perfusion tubes. Comparisons showed no differences between the procedures when perfusing with standard 300-mOsm solution. Effluent samples were collected from outlet tubes at 20-min intervals during the 5-h period of the experiment. When concentrations or osmolalities were measured, samples were accumulated under mineral oil. One hour after labeling with $^{14}CO_2$, the labeled portion of the flag leaf was cut off. This generated a sharp pulse of ^{14}C-translocate without affecting solute concentration in the sieve tubes or the translocation rate into the ear (Fisher, 1990; Fisher and Wang, 1993).

In experiments to determine the long-term effects of perfusion, grains were perfused with 300 mOsm standard perfusion solution for 24 h before labeling with $^{14}CO_2$.

Assays

After terminating an experiment, the perfused grains and four control grains (intact A or B grains from the same spikelets as the perfused grains) were removed from the ear. Cavity sap was collected from control grains. The "crease tissues" (i.e. the vascular strand, chalaza, nucellus, and surrounding pericarp) were dissected from each grain and heated in 1 mL of water at 100°C for 2 min to inactivate invertase. (In Suc perfusion experiments, crease tissue samples were first rinsed for 3 min in standard perfusion medium to remove free-space Suc.) Crease and endosperm samples were homogenized with 1 mL of water, centrifuged, and assayed for Suc, Glc, and Fru (Jones et al., 1977), and for ^{14}C. The insoluble portion of the endosperm was suspended and recentrifuged three times with 4-mL aliquots of water and heated at 100°C for 2 h in 1 mL of water. The gelatinized starch was hydrolyzed with amyloglucosidase at 55°C for 5 h for ^{14}C counting.

Effluent osmolalities were measured by a nanoliter osmometer (Clifton Technical Physics, Hartford, NY). A liquid scintillation counter was used to assay ^{14}C. Enzymes for sugar assays were purchased from Boehringer Mannheim Biochemicals. Other chemicals were from Sigma.

Measurement of Assimilate Import Rate

Two approaches were taken to the measurement of assimilate import into perfused grains. The first was the *RUR*, defined as:

$$RUR = \frac{\text{Total } ^{14}\text{C entering a perfused grain}}{\text{Average of total } ^{14}\text{C in 4 control grains}} \quad (1)$$

Total ^{14}C entering a perfused grain was the sum of ^{14}C collected in the effluent solution plus that remaining in the grain. Respiratory $^{14}CO_2$ was ignored in this and other calculations, but amounted to less than 5% of the ^{14}C entering the grain (data not shown).

The second approach to calculating import rate was the *SRR*, an estimate of the rate of Suc movement into the endosperm cavity from the nucellus. This was based on the assumption that, after Suc efflux from the endosperm cavity reached a steady state, the partitioning of Suc between retention in the grain and outflow via perfusion would be the same as the partitioning of ^{14}C, that is:

$$\frac{\text{Suc efflux}}{SRR} = \frac{\text{Total effluent } ^{14}\text{C}}{(\text{Total grain } ^{14}\text{C} - \text{Crease } ^{14}\text{C})} = \text{Partitioning ratio} \quad (2)$$

or

$$SRR = \frac{\text{Suc efflux}}{\text{Partitioning ratio}} \quad (3)$$

As will be noted later, other assumptions must also apply for Equation 3 to provide an accurate measure of the *SRR*. That is, the partitioning ratio should be fairly constant throughout the experiment and, at steady state, only Suc released from the nucellus should contribute to Suc in the effluent.

In calculating SD values, each grain was treated as an

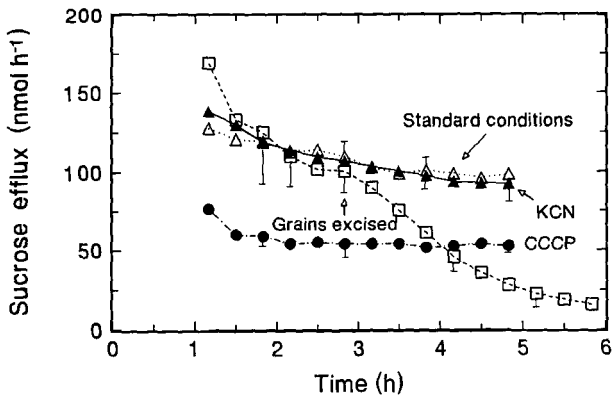

Figure 2. Suc efflux from the endosperm cavity of perfused grains. Standard conditions, Perfused at 6 μL h^{-1} with 300 mOsm perfusion medium (n = 4 grains). CCCP, Same as standard conditions, plus 250 μM CCCP (n = 4 grains). KCN, Same as standard conditions, plus 5 mM KCN (n = 4 grains). Grains excised, Same as standard conditions; grains were detached from the plant at the time indicated (two experiments, n = 8 grains).

independent sample (thus, each experiment consisted of four samples). This is justified by the relative independence of (i.e. absence of interactions between) individual grains of an ear during the linear phase of grain filling (Brocklehurst, 1977; Fischer and Hille Rislambers, 1978; Jenner, 1980). Values for *RUR* and *SRR* from replicate experiments were combined, since their means were rarely different ($P > 0.4$) and coefficients of variation were rarely more than 25%.

RESULTS

RUR and *SRR* under Standard Perfusion Conditions

Examples of the time course for Suc efflux are shown for several experimental treatments in Figure 2. Efflux declined during the first 2 h of perfusion due to the initially higher concentration of Suc in the cavity. Subsequently, it remained essentially constant even during inhibitor or osmotic treatments. In the example experiment run under "standard perfusion conditions" (i.e. using 300 mOsm perfusate), the mean Suc efflux (average of final 3 h) for the four grains was 105 ± 6 nmol h^{-1} $grain^{-1}$ (effluent Suc concentration about 18 mM). With a partitioning ratio of 0.40 ± 0.05 (n = 4), the calculated *SRR* was 262 nmol h^{-1} $grain^{-1}$ (105 nmol h^{-1} $grain^{-1}$/0.40). This compares with the expected rate, based on the rate of grain filling, of 220 nmol h^{-1} $grain^{-1}$ (Fisher, 1990). The *RUR* in this experiment was 1.23 ± 0.12. The Suc pool size in the crease tissues of grains was unchanged compared with that of intact grains (Table I).

Under standard perfusion conditions and in other mannitol perfusion experiments, the kinetics of Suc labeling were reproducible from experiment to experiment, at least in terms of relative specific activity, and similar to the kinetics reported earlier for the endosperm cavity (Fisher and Wang, 1993; see also Fig. 3). On average, ^{14}C was initially detected about 1 h after labeling, and Suc specific activity reached a maximum

Table I. *Crease ^{14}C, crease Suc pool size, and SRR for various perfusion experiments*

Perfusion Treatment[a]	No. of Grains	Crease ^{14}C	Crease Suc		SRR	Effluent Suc
		% total	*nmol*	*% control*[b]	*nmol h^{-1} $grain^{-1}$*	*mM*
Intact grains (not perfused)	108	17 ± 3	299 ± 70	100	220[c]	NA[d]
29-h perfusion	7	17 ± 2	322 ± 26	95	208 ± 18	13 ± 2
Grains excised	8	NA	39 ± 10	13	NA	NA
Buffer and salts only (30 mOsm)	5	ND[d]	213 ± 27	92	178 ± 18	14 ± 2
Mannitol						
300 mOsm	10	16 ± 4	334 ± 90	100	248 ± 20	18 ± 3
600 mOsm	8	20 ± 1	206 ± 18	112	225 ± 9	16 ± 3
910 mOsm	7	ND	ND	ND	260 ± 6	23 ± 3
1110 mOsm	9	13 ± 2	207 ± 25	100	216 ± 35	17 ± 6
Suc						
1105 mOsm	4	22 ± 3	630 ± 19	186	NA	632[e]
1490 mOsm	6	25 ± 11	452 ± 91	155	NA	740[e]
2820 mOsm	4	20 ± 4	ND	ND	NA	980[e]
Sorbitol						
1150 mOsm	4	68 ± 11	369 ± 38	144	205 ± 32[f]	7 ± 4
1660 mOsm	3	71 ± 4	514 ± 15	174	450 ± 304[f]	3 ± 1
CCCP						
0.25 mM	4	20 ± 2	210 ± 33	75	95 ± 9	9 ± 1
KCN						
5.0 mM	8	22 ± 2	187 ± 16	63	210 ± 18	18 ± 2

[a] All experiments lasted 5 h except for the extended perfusion experiment (29 h). [b] Based on the crease Suc pool of nonperfused grains from the same ear. [c] Calculated from the grain-filling rate (Fisher, 1990). [d] NA, Not applicable; ND, not determined. [e] Calculated from the average effluent osmolality of Suc (see Fig. 5). [f] Spuriously high due to low partitioning ratios (see Eqs. 2 and 3).

about 1.5 h later. It then declined to about 30% of maximum activity 5 h after labeling, at which time the experiment was usually terminated.

To establish more firmly the direct relationship between assimilate import into the grain and Suc efflux from the cavity, we followed the effect of grain excision on Suc efflux (Fig. 2). Efflux from the endosperm cavity dropped immediately after the grains were excised, declining in 3 h to 16% of the preexcision rate. The crease Suc pool declined to 13% of that in control grains (Table I).

Phloem unloading and post-phloem transport continued strongly even after 29 h of continuous perfusion (Fig. 3). The *SRR* in two experiments was 208 ± 18 nmol h^{-1} $grain^{-1}$ (n = 7), and the *RUR* was 0.91 ± 0.07. The crease Suc pool was unaffected by the extended perfusion (Table I).

The *RUR* was consistently greater than 1.0 under standard perfusion conditions. In the light, however, ear photosynthesis supplies about 35% of the assimilates for grain filling (Fisher, 1990). We reasoned that this source of unlabeled assimilates might be greatly decreased by cutting away the ends of glumes around an experimental grain, since the enclosed environment around the grain is opened and much of the glume chlorenchyma is excised. The grains would then be more dependent on ^{14}C-assimilates arriving via the peduncle. This appeared to be the case, since the *RUR* of unperfused grains was 1.26 ± 0.32 (n = 15) when the only surgery performed was to remove the ends of their surrounding glumes.

The *SRR* was also often somewhat higher than the expected rate of 220 nmol h^{-1} $grain^{-1}$ (Fisher, 1990). Since a backflux of unlabeled Suc from the endosperm could account for a spuriously high *SRR*, we surveyed the relationship between initial cavity sap Suc concentration and *SRR* (Fig. 4) on the premise that a backflux might result in a higher steady-state cavity Suc concentration. There was a significant positive correlation ($P < 0.01$) between *SRR* and cavity Suc concentration of intact grains (presumed to indicate initial concentrations of the perfused grains; Fisher and Gifford, 1986).

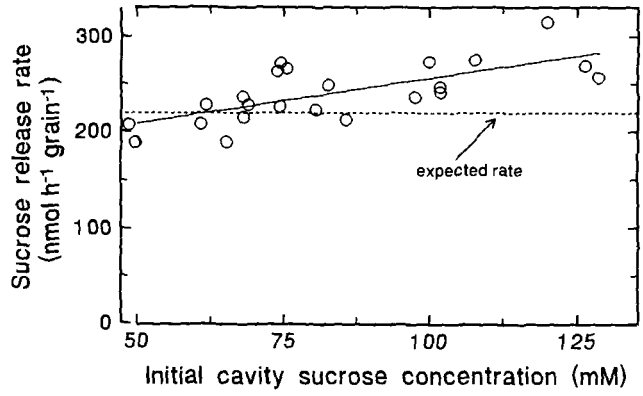

Figure 4. Correlation between initial cavity Suc concentration, estimated from intact grains, and *SRR* (r = 0.76). The expected Suc import rate, based on the rate of grain filling, is 220 nmol h^{-1} $grain^{-1}$ (Fisher, 1990).

Despite the low perfusion rate, efflux osmolality was quite stable after the 1st h, with the exception of 2820 mOsm Suc perfusate. The osmolality of standard 300-mOsm perfusion medium was unchanged after passing through the endosperm cavity, whereas solutions of higher osmolality were reduced in concentration, with the extent of the reduction increasing with increasing osmolality. Effluent from the perfusate of lowest osmolality (buffer and salts only) was slightly increased in concentration, from 30 to 90 mOsm. (It should be noted that, since the effluent provides a continuous sampling of the cavity contents, effluent concentration, osmolality, etc. are more relevant measures of treatment conditions than the entering perfusate.)

For all three sugars, the *RUR* was not noticeably affected, if it was affected at all, by effluent osmolalities below about 500 mOsm. At higher concentrations, *RUR* declined progres-

Effect of Osmotic Treatments

The response of the *RUR* to perfusion with various concentrations of Suc, mannitol, and sorbitol are shown in Figure 5.

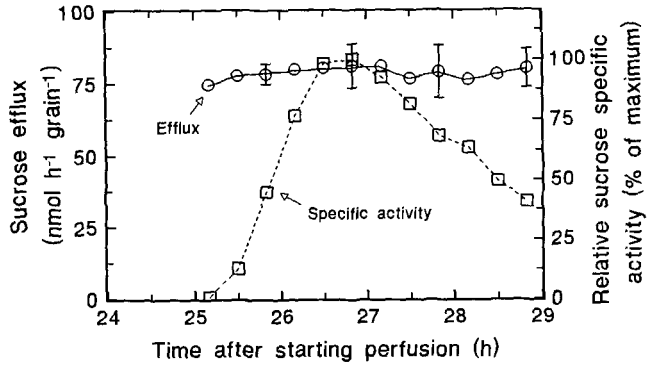

Figure 3. Suc efflux and relative Suc specific activity during two long-term perfusion experiments (29 h). Grains were perfused for 24 h before the flag leaf was labeled with $^{14}CO_2$ (n = 7 grains).

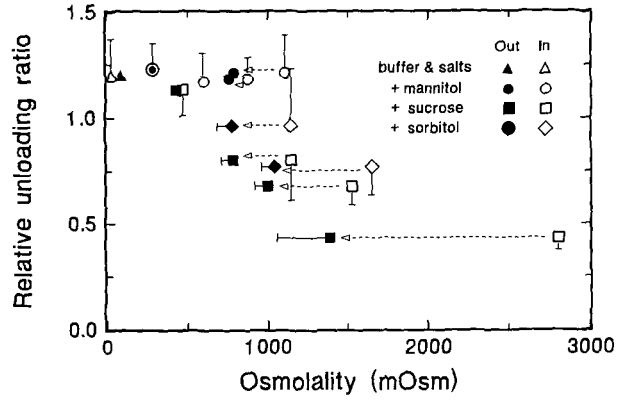

Figure 5. Effect on the *RUR* of perfusing the endosperm cavity with various concentrations of mannitol, Suc, or sorbitol. Arrows indicate the change in osmolality between the perfusing solutions (open symbols) and effluent solutions (solid symbols). Horizontal error bars indicate the variation in effluent osmolality during the experiment.

sively with increasing effluent Suc and sorbitol concentrations. However, although experiments with these solutes are grouped for convenience as "osmotic treatments" (and this was their original intent), their effects were not equivalent, nor were they necessarily "osmotic."

Even at the highest concentrations used, mannitol had no apparent effect on phloem unloading or post-phloem transport of Suc in perfused grains. This is evident not only in the *RUR* (Fig. 5), but in the lack of effect on *SRR*, crease Suc pool size, and the proportion of ^{14}C remaining in the crease tissues at the end of the experiment (Table I).

In Suc-perfusion experiments, the *RUR* declined progressively with effluent concentrations above about 500 mOsm. The reduction clearly contrasted to the effect of mannitol perfusion, since, at nearly equal mean effluent osmolalities for Suc and mannitol (790 versus 780 mOsm, respectively), the *RUR* for Suc perfusion (0.78 ± 0.19, $n = 8$) was significantly less ($P < 0.005$) than for mannitol perfusion (*RUR* = 1.20 ± 0.19, $n = 7$). The decline in *RUR* with increasing Suc concentration was not pronounced, however. Even at an effluent osmolality of about 1000 mOsm, close to that found in the sieve tubes of these plants (Fisher, 1990), the *RUR* was still 0.68.

The crease Suc pool was substantially increased in Suc-perfused grains (Table I). The increase cannot be attributed to free-space Suc, since the nucellar walls, the only free space in the crease available for exchange with the endosperm cavity (Wang and Fisher, 1994), accounted for less than 10% of the crease volume and most Suc would have been lost from there in the 3-min wash used (Wang and Fisher, 1994). Blockage of release from the nucellus cannot be the cause of the increased Suc pool, since almost 80% of the [^{14}C]Suc, a proportion comparable to that for intact grains, passed through the crease into the cavity (Table I).

Unloading and post-phloem movement into the endosperm were fairly even along the length of the grain (Table II). Thus, possible effects arising from incomplete solute mixing within the cavity appear to be minor. In this context, it should be noted that, although the distance between inlet and outlet perfusion tubes was only 2 to 3 mm, diffusion alone should provide an effective basis for mixing for 1 to 2 mm beyond the end of each tube, given the time scale of these experiments. The possibility of water potential gradients along the crease tissues should be minimized further by the presence of xylem vessels there.

To provide a non-Suc treatment with an osmolality higher than that available with mannitol, a high concentration of sorbitol (perfusate = 1660 mOsm; mean effluent osmolality = 1040 mOsm) was used as osmoticum. A sorbitol perfusate of 1150 mOsm (mean effluent osmolality = 780 mOsm) was also used to provide a comparison with one of the Suc treatments and with mannitol at its highest concentration. The effects of sorbitol on *RUR* approximated those of Suc at similar osmolalities (Fig. 5) and, like Suc, the *RUR* for sorbitol was reduced in comparison to that for mannitol at a similar osmolality. However, in contrast to both mannitol and Suc, the movement of Suc into the endosperm cavity was sharply reduced by the sorbitol solution (partitioning ratio = 0.15 ± 0.14, versus 0.40 ± 0.05 in grains under standard perfusion conditions). At the higher sorbitol concentration, almost no ^{14}C entered the endosperm cavity (partitioning ratio = 0.04 ± 0.02). Instead, much of the imported [^{14}C]Suc remained in the crease tissues, resulting in a sharp increase of the Suc pool there (Table I).

The possible extent of plasmolysis in the crease tissues was examined in grains perfused for 2 h with a high concentration of Suc (attached grains) and for 5 h with high concentrations of Suc (attached and excised grains), mannitol, or sorbitol (attached grains). Although preliminary experiments with 100-μm-thick fresh cross-sections had shown that all of the crease tissues were plasmolyzed at or above 520 mOsm Suc, this proved to be a poor guide to the response of cells in perfused grains. When attached grains were perfused with high mannitol or Suc concentrations (mean effluent osmolality = 800 and 970 mOsm, respectively) (Fig. 6, A and C) none of the crease cells, even in the nucellus, were plasmolyzed. No difference was noted between the 2-h and 5-h Suc-perfused grains. However, when excised grains were perfused for 5 h with 1490 mOsm Suc (mean effluent osmolality = 1000 mOsm), nucellar cells were clearly plasmolyzed, chalazal cells were more so, and vascular parenchyma cells were strongly shrunken rather than plasmolyzed (Fig. 6D). The response of the crease tissues in attached grains perfused with 1660 mOsm sorbitol (mean effluent osmolality = 1040 mOsm) was particularly interesting (Fig. 6B). Here, the nucellar cells were strongly plasmolyzed, there was a gradient in plasmolysis from strong to none across the chalaza, and vascular parenchyma cells were neither plasmolyzed nor shrunken.

Table II. *Uniformity of phloem unloading along the crease tissues of wheat grains after perfusion of the cavity with 1490 mOsm Suc or with 2 mM KCN*

Crease samples were obtained during the 20- to 25-min interval after a readily countable level of ^{14}C had appeared in the grains, as determined by periodically sampling intact grains. ^{14}C in the grain during this interval would be almost entirely unloaded from the phloem but still present in the crease Suc pool (Fisher and Wang, 1993). The crease tissues were then cut into thirds, homogenized, and assayed for ^{14}C.

Perfusion Treatment	No. of Grains	Base	Middle	Apex
		% total ^{14}C		
Suc (1490 mOsm)				
Perfused	4	34 ± 1	34 ± 3	32 ± 1
Intact	4	35 ± 2	36 ± 1	29 ± 1
KCN (2 mM)				
Perfused	2	33 ± 0	34 ± 2	35 ± 2
Intact	2	29 ± 5	36 ± 3	35 ± 4

Effects of Respiratory Inhibitors

The *RUR* declined with increasing concentrations of both CCCP (Fig. 7) and KCN (Fig. 8). At inhibitor concentrations (250 μM CCCP and 5 mM KCN) where the *RUR* was about 0.3 for each, the crease Suc pool was reduced similarly by both inhibitors, to about 70% of control grains (Table I). The conversion of [^{14}C]Suc into starch was almost completely

blocked by both inhibitors (2% incorporation into starch versus 15% in nonperfused grains or in grains perfused with standard medium). However, there was a marked difference in their effect on *SRR*, which declined in parallel with *RUR* in CCCP treatments (Fig. 7) but was affected only slightly by KCN (Fig. 8). This difference was immediately evident in the Suc efflux from perfused grains (Fig. 2; see also the efflux concentrations, Table I), which were reduced by CCCP perfusion, but not by KCN. Evidently KCN, but not CCCP, caused endosperm cell membranes to become leaky. The effect of KCN on phloem unloading was uniform along the length of the grain (Table II).

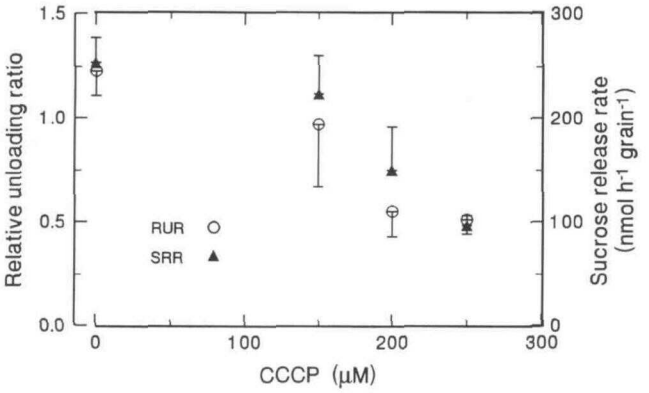

Figure 7. Effects of CCCP on *RUR* and *SRR*.

DISCUSSION

Perfusion Did Not, in Itself, Alter Transport within the Crease Tissues

Phloem unloading and post-phloem transport in developing wheat grains appeared to be unaffected by the perfusion procedures applied. Under standard perfusion conditions, *RUR* and *SRR* differed only marginally, if at all, from in vivo transport rates. Even after 29 h of continuous perfusion, *RUR* and *SRR* continued at close to expected rates. Crease Suc pool sizes were unchanged in both 5- and 29-h perfusion experiments, indicating that the component transport steps within the maternal tissues were unaffected by perfusion. Finally, although *RUR* was often higher than 1.0, this did not result from a stimulation of import into experimental grains, but from a compensatory shift from the partial import of glume photosynthate to essentially complete dependence on peduncle-imported assimilates.

Although we chose 6 μL h^{-1} as our standard perfusion rate, slow perfusion was not a critical condition for avoiding effects on transport. *RUR* and *SRR* were unaffected over 6 to 18 μL h^{-1} (data not shown). However, high perfusion rates sometimes caused leakage due to increased pressure in the cavity.

Transport Was Unaffected over Physiologically Relevant Ranges of Cavity Sap Osmolality and Suc Concentrations

Compared with normally existing conditions in the endosperm cavity, Suc import into the grains was unaffected over much wider ranges of osmotic and Suc effluent concentrations. Under our growth conditions, cavity sap osmolality

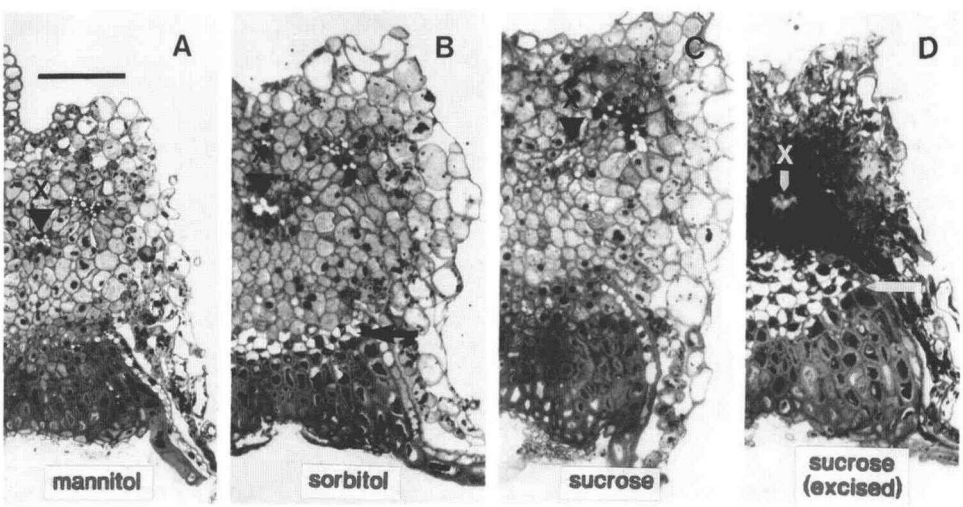

Figure 6. Appearance of the crease tissues in attached (A–C) or excised (D) grains perfused with high concentrations of sugars for 5 h. Sections 2 μm in thickness were stained with toluidine blue O. For orientation, the position of the xylem (X) is indicated in each micrograph. Bar = 100 μm. A, Attached grain perfused with 1110 mOsm mannitol medium (mean effluent osmolality = 780 mOsm). No plasmolyzed cells were observed. B, Attached grain perfused with 1660 mOsm sorbitol medium (mean effluent osmolality = 1040 mOsm). A band of strongly plasmolyzed chalazal cells is present (horizontal arrow). Some nucellar cells are also plasmolyzed. C, Attached grain perfused with 1490 mOsm Suc medium (mean effluent osmolality = 960 mOsm). No plasmolyzed cells were observed. D, Excised grain perfused with 1490 mOsm Suc medium (mean effluent osmolality = 1000 mOsm). Most of the chalazal cells are plasmolyzed (horizontal arrow), whereas the vascular parenchyma around the xylem has shrunken as a unit (cytorrhysis).

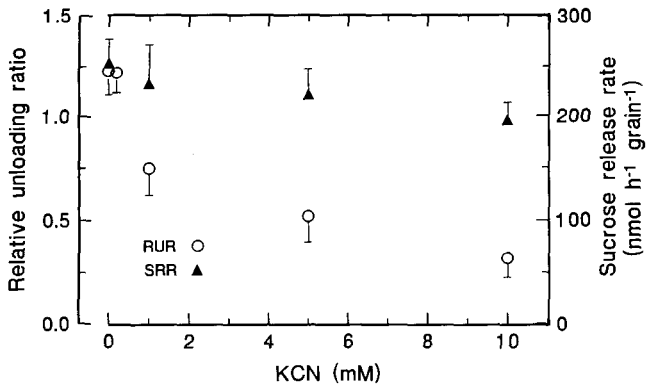

Figure 8. Effects of KCN on *RUR* and *SRR*.

ranged from 230 to 300 mOsm (Fisher and Gifford, 1986; D.B. Fisher, unpublished data), and Suc concentrations ranged from 40 to 120 mM (Fisher and Wang, 1993). The *RUR* and *SRR*, however, were unaffected over 90 to 500 mOsm effluent osmolality (Fig. 5 and Table I). Similarly, the *RUR* was unaffected over 14 to 500 mOsm (14–430 mM) Suc, nor, judging from the proportion of ^{14}C in the crease, did there appear to be a strong effect of Suc concentration on post-phloem movement (Table I and Fig. 5). Previously, Fisher and Gifford (1987) concluded that the growth rate of wheat grains was not responsive to short-term variations in cavity sap Suc concentration.

For the most part, our observations strongly reinforce the view that the rate of import into developing seeds is regulated primarily by transport processes within the maternal tissues. As Wolswinkel (1992) points out for the empty seed coat and empty corn pedicel systems (Porter et al., 1985), their ability, under proper conditions, to sustain in vivo transport rates argues against a direct involvement of embryonic tissues in controlling import. This is supported more emphatically by our present observations, since import not only continued at or near in vivo rates, but was unaffected over much broader ranges of Suc and osmotic concentrations than occur normally within the endosperm cavity. Nonetheless, our results do not rule out possible effects of embryonic tissues having a time delay of several hours or more.

Based on his own extensive work and that of others, Jenner (1985a, 1985b), as well, has argued that the control of wheat grain growth lies in the grain itself. He considers as candidates two transport pathways in the maternal tissues, one across the crease tissues (Jenner, 1985a) and the other in the region of the xylem discontinuity (Jenner, 1985b). Both may be important. Although our present observations emphasize phloem unloading and post-phloem movement across the crease, the relative independence of grain water relations conferred by the xylem discontinuity can be expected to have an important impact on phloem water relations within the grain.

The relative insensitivity of assimilate import to apoplastic solute concentration, especially over physiologically relevant ranges (i.e. to about −1 MPa; Wolswinkel, 1992), contrasts with the response of legume seed coats (Patrick, 1990; Wolswinkel, 1990, 1992; Ellis et al., 1992) and corn pedicels (Porter et al., 1987). Although those systems react differently to increasing osmotic concentration (solute release from seed coats increases, whereas that from pedicels decreases), both appear to be responding to osmotically induced turgor changes in the maternal tissues. However, the interpretation of osmotic treatments in wheat grains differs in several respects from that in legumes and corn, particularly in the presence of two barriers to apoplastic solute (and water?) movement, one in the chalazal cell walls (Wang and Fisher, 1994) and another at the base of the grain (Zee and O'Brien, 1970a). It is unclear how effectively changes in cavity water relations might be transmitted to the vascular parenchyma or crease phloem or, given the relative independence in grain (and crease?) water relations that is evidently conferred by the xylem discontinuity (Barlow et al., 1983; Fisher, 1985), how much their turgor might be affected. Also, given the intrinsically high solute fluxes of the post-phloem transport pathway, substantial osmotic adjustments could occur quite rapidly. Clearly, some measurements of concentrations and/or turgor pressures are needed to clarify these issues.

Perfusion with Sugars Reduced Assimilate Import, but Only at High Concentrations

Although originally conceived as "osmotic treatments," perfusion of the endosperm cavity with high sugar concentrations provoked responses that depended strongly on the particular sugar involved. Of the three used, only sorbitol appeared to elicit an essentially "osmotic" response.

Mannitol had no evident effect on any transport-related measurement (i.e. *RUR*, *SRR*, crease Suc pool, ^{14}C distribution) over the entire range of effluent osmolalities (up to 790 mOsm). Furthermore, there was no plasmolysis in the crease tissues after the cavity had been perfused for 5 h with the highest concentration of mannitol (mean effluent osmolality = 790 mOsm). Possibly, early plasmolysis was followed by recovery. However, judging from the pronounced blockage of transcrease movement caused by sorbitol-induced plasmolysis, the duration of any plasmolysis must have been brief. The absence of plasmolysis after 5 h suggests that the nucellar cell membrane may be fairly permeable to mannitol. This possibility is supported by microautoradiographic observations showing that [^{3}H]mannitol, applied for 8 h to the nucellus of an excised grain, had penetrated all cells of the crease tissues, including the pericarp (observations not shown).

Suc and sorbitol substantially reduced assimilate import only at effluent osmolalities comparable with that of the sieve tube contents (about 800 mOsm or higher). Although the immediate effects of the two sugars differed, each caused the crease Suc pool, located mostly in the vascular parenchyma (Fisher and Wang, 1993), to increase. Sorbitol, by plasmolyzing the nucellus and part of the chalaza, caused a buildup of phloem-imported Suc. On the other hand, perfusion with Suc did not cause plasmolysis in attached grains, nor did it cause a buildup of imported Suc in the crease tissues. Instead, the nucellar cell membranes appear sufficiently permeable to Suc to allow substantial movement from the cavity into the vascular parenchyma. Thus, the increased Suc level there

may have increased the turgor pressure during perfusion with both Suc and sorbitol. Since phloem unloading in the wheat grain is symplastic (Wang and Fisher, 1994) and presumably turgor driven (Murphy, 1989), increased turgor in the vascular parenchyma should slow phloem unloading. As previously noted, however, the effect of cavity perfusion on crease water relations is uncertain. Also, if the scenario is correct, it is unclear why mannitol perfusion would not also inhibit phloem unloading.

The patterns of plasmolysis and cytorrhysis observed under some perfusion conditions are not entirely explainable from our limited observations. However, they may have significant implications for normal solute and water movement across the crease tissues. Plasmolysis of nucellar cells appeared to occur "normally," i.e. solution flowed through the walls as the protoplast receded. In the chalaza, however, the strong restriction placed on solute movement in the walls (Wang and Fisher, 1994) may require solution flow via an alternative route during protoplast shrinkage. Potentially, plasmolysis could provide this route by breaking and/or withdrawing plasmodesmata from pit fields between adjacent cells, eventually allowing the cell-to-cell movement of solution. Slow movement of solution by either pathway would presumably result, at any given time, in a sharp boundary between plasmolyzed and nonplasmolyzed cells in the chalaza. In attached grains, this process appears to have been slowed (sorbitol-perfused grains) or prevented (Suc-perfused grains) by the continued replenishment of solutes and water from phloem unloading into the vascular parenchyma cells. In an excised grain, the absence of unloading would lead to net water and solute loss from the crease tissues. However, it is unclear why the pattern of cell water loss changed from plasmolysis to cytorrhysis at the boundary between chalazal and vascular parenchyma cells. In any event, the wholesale shrinkage of the vascular parenchyma in excised grains suggests a process of symplastic dehydration in which both solutes and water moved through plasmodesmata into the chalaza. Net flow via the symplast may or may not occur normally along this path, but it appears at least potentially capable of accommodating such movement.

The Effects of KCN, CCCP, and High Suc Concentrations Suggest, Inconclusively, the Occurrence of Active Transport across Nucellar Cell Membranes

The inhibition of Suc import by KCN and CCCP, and the apparent movement of Suc against its concentration gradient (approximately 600 mM in the sieve tubes versus >700 mM in the cavity in some experiments), suggest the existence of an active transport step somewhere along the intervening pathway. Presumably, this would occur at the nucellar cell membranes, since the rest of the pathway is entirely symplastic. Nonetheless, although the *RUR* was reduced by both inhibitors, the crease Suc pool also declined, suggesting that phloem transport and/or unloading was more strongly affected than post-phloem symplastic movement and/or solute release into the endosperm cavity. In addition, the increased pool size of Suc-perfused grains suggests that movement across nucellar cell membranes was reversible. Thus, although the basis for their effect is unclear, the action of KCN and CCCP within the crease appeared to be similar and not proximally related to their site of application (i.e. the nucellus).

The occurrence of pressure-driven assimilate flow in the wheat grain may account for the movement of Suc against its concentration gradient. Clearly, bulk flow is the basis for movement along the sieve tubes and, almost certainly, for unloading from the sieve tube-companion cell complex (Murphy, 1989). As noted, bulk flow may also contribute to symplastic movement across the crease, especially during perfusion with concentrated solutions. Assimilates could be concentrated at sites where water was withdrawn from cells more rapidly than solutes. Thus, the evidence for an active transport step is inconclusive.

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